THE UNIVERSITY OF MANITOBA

" THE EFFECT OF NIACIN DEFICIENCY ON THE REGULATION OF PYRIDINE NUCLEOTIDES AND ENZYMES IN VARIOUS TISSUES OF JAPANESE QUAIL "

BY

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ABSTRACT

The addition of 0.5% sulfaguanidine or high leucine to niacin free diets did not induce niacin deficiency symptoms in mature Japanese quail. Niacin deficiencies in growing quail resulted in the typical growth depression, poor feather development, blindness, and lowering of NAD and NADPH levels in the breast muscle. However, there were no significant differences in pyridine coenzyme levels in liver, brain, and heart as compared to niacin fed quail. Similarly, there were no significant differences in activities of liver aldolase, lactic dehydrogenase, glutamic dehydrogenase, tryptophan pyrrolase, fructose diphosphatase, and malic enzyme. Niacin deficiency or food-restriction led to a reduction in breast muscle lactic dehydrogenase or glyceraldehyde-3-phosphate dehydrogenase activity.

6-Aminonicotinamide as an antagonist of niacin did not affect the levels of pyridine coenzyme, ATP, lactic dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase activity in liver or breast muscle whereas specific activities of liver malic enzyme and fructose diphosphatase of the control group were significantly higher than those of the 6-aminonicotinamide treated group.

The apparent turnover rates of radioactive products in breast muscle following the injection of 7-^{14}C-nicotinic acid were extremely low while the turnover rates in liver were much higher than those in breast muscle. Turnover rates in

heart and brain were intermediates to those of liver and breast muscle. The apparent turnover rates of niacin products in all tissues in the niacin deficient quail were the slowest whereas those of quail fed a high level of niacin were the fastest. The apparent half-lives of nicotinamide, NAD, and NADP in liver of the niacin deficient quail were estimated to be 80.3, 100.2, and 626.0 hours, respectively, whereas the corresponding values for the food-restricted quail were 92.1, 87.7, and 469.2 hours, respectively. In breast muscle the apparent half-life of nicotinamide in the food-restricted quail was about 10 times shorter than that of the niacin deficient quail. The apparent half-life of NAD in the food-restricted quail was 490.1 hour whereas the corresponding value was infinite in the niacin deficient quail.

Thermal stability studies on liver and breast muscle enzymes from control, niacin deficient and food-restricted quail showed that there were no significant differences in the thermal stability of liver malic enzyme, glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase,and aldolase among treatments. Similarly, no significant differences in thermal stability of breast muscle lactic dehydrogenase and aldolase were observed. However, breast muscle glyceraldehyde-3-phosphate dehydrogenase activity in niacin deficient quail was significantly reduced as compared to that from the control and food-restricted quail. Among all of the liver and breast muscle enzymes tested, breast muscle glyceraldehyde-3-phosphate dehydrogenase appeared to be the most

sensitive of all enzymes to heat inactivation in the absence of NAD or NADP in the incubation mixture.

The effects of niacin deficiency on relative turnover rates of proteins showed that liver had the fastest turnover rates, that heart and brain had the intermediate turnover rates and breast muscle had the slowest turnover rates of proteins irrespective of dietary treatments. The $^{3}H/^{14}C$ ratios of both control and niacin deficient quail were significantly higher than those of the food-restricted quail but there were no significant differences between the former groups. This suggests that the relative turnover rates of proteins in the niacin deficient quail were much greater than those of the food-restricted quail. The resolution of breast muscle proteins by isoelectric focusing showed greater degradation of certain specific acidic proteins in niacin deficient quail relative to control quail.

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ABBREVIATIONS

A_{260}: Absorption at 260nm of a solution in a cuvette with a one centimeter lightpath.

ADH: Alcohol dehydrogenase.

ADP: Adenosine-5'-diphosphate.

ADRP: Adenosine diphosphoribose.

AMP: Adenosine-5'-monophosphate.

6-AN: 6-Aminonicotinamide.

ATP: Adenosine-5'-triphosphate.

CoA: Coenzyme A.

DNA: Deoxyribonucleic acid.

DEAE: Diethylaminoethyl.

EDTA: Ethylenediaminetetraacetic acid.

G.I.Tract: Gastrointestinal tract.

GSH: Reduced glutathione.

IDH: Isocitrate dehydrogenase.

IU: International unit.

LDH: Lactic dehydrogenase.

NAD: Nicotinamide adenine dinucleotide.

NADP: Nicotinamide adenine dinucleotide phosphate.

NFD: Niacin free diet.

O.D.: Optical density.

Pi: Inorganic phosphate.

PMSF: Phenylmethylsulfonyl fluoride.

Poly U: Polyuridylic acid.

POPOP: 1,4 bis-{2-(5-phenyloxazoyl)} benzene.

PPi: Pyrophosphate.

PRPP: 5-phosphoribosyl-1-pyrophosphate.

RNA: Ribonuleic acid.

mRNA: Messenger RNA.

tRNA: Transfer RNA.

SDS: Sodium dodecyl sulphate.

TCA: Trichloroacetic acid.

Tris: Tris(hydroxymethyl) aminomethane.

INTRODUCTION

Niacin is a precursor of pyridine coenzymes. They have been implicated in most of the major metabolic pathways. As the prosthetic group of several dehydrogenases, they are involved in glycolysis, fat synthesis and tissue respiration. Recently NAD has been shown to have functions beyond its well known role as an electron carrier in various biological oxidation-reduction systems.

One such area is that NAD is consumed in certain metabolic reactions in which it serves as a substrate. In eukaryotic cells, the most intriguing of these reactions is the cleavage of NAD to form nicotinamide and a unique polymer, poly adenosine diphosphoribose (poly ADPR); the reaction is catalyzed by the enzyme poly ADPR synthetase (Chambon et al., 1963). Other well studied reactions involving the destruction of NAD include the cleavage of NAD to form AMP and NMN (nicotinamide mononucleotide) by bacterial DNA ligases (Olivera and Lehman, 1967) and the breakdown of NAD (with the concomitant inactivation of protein synthesis) by the diphtheria toxin (Collier and Pappenheimer, 1964). Thus, one could envision a rather high turnover of NAD in various cells and this emphasizes the importance of reutilizing nicotinamide in an efficient manner.

The objective of this thesis was to investigate in greater detail the development of niacin deficiency and its effect on turnover of pyridine coenzymes and to establish

the role which niacin plays in the turnover of tissue proteins, particularly those proteins that have a coenzyme requirement for pyridine nucleotides.

More specific objectives were:

1. To establish the reliability of methods for the extraction and determination of pyridine nucleotide contents. This included:
   a) the recovery of pyridine nucleotides in the extraction medium and assay mixture.
   b) the stability of pyridine nucleotides as affected by post-mortem changes, duration of homogenization time and storage in the presence of reducing reagents.
2. To induce niacin deficiency in mature female quail (127 - 128 g). This included a study on the effect of high leucine or sulfaguanidine in a niacin free diet on growth, liver weight, liver pyridine nucleotide content and liver enzyme activity levels.
3. To induce niacin deficiency in immature quail (55 - 62 g) in a manner similar to that outlined above.
4. To determine the effect of niacin deficiency in growing quail of varying ages. The response in growth, pyridine nucleotide contents and enzyme activity levels to graded levels of nicotinic acid and to a high level of tryptophan in a niacin-free diet was followed.
5. To determine if 6-aminonicotinamide (6-AN), as an anti-niacin, produced a pattern of symptoms or metabolic dis-

turbances similar to those seen in the niacin deficient birds. This included the effect of 6-AN on growth, organ weights, pyridine nucleotide and ATP levels, and enzyme activity levels.

6. To compare apparent turnover rates of radioactive niacin metabolites in various tissues in response to different dietary treatments. This included calculation of apparent half-lives of radioactive NAD, NADP, and nicotinamide.
7. To determine if the in vitro stability of liver or breast muscle enzymes from the niacin deficient and food-restricted (but nicotinic acid supplemented) quail was affected by the concentration of pyridine nucleotides.
8. To determine if niacin deficiency altered the rate of protein turnover in various tissues of immature quail. This included the effect of subunit size and isoelectric points on relative turnover rates of breast muscle proteins.

LITERATURE REVIEW

1. NIACIN

Discovery

The search for the cause of pellagra forms a fascinating Chapter in medical history. The disease was first described by Casal (1762) in northwestern Spain in 1730 under the name mal de la rosa. It occurred among the poor peasants for whom corn was the chief article of the diet. In 1771 Frapolli described a syndrome in the country districts of Italy which was known as pellagra from the words pella, skin and agra, unsightly or rough.

Early observers of pellagra noted that the syndrome appeared to spread throughout southern Europe in conjunction with the use of maize in the diet. One of the theories of the etiology of the disease was that it was due to an insufficient or badly balanced diet. In 1795 in Italy, Cerri demonstrated the importance of diet in the production of pellagra. In the long search for the cause of pellagra, a close association with the eating of maize was emphasized repeatedly.

In the early 1800's, Buniva carried out a series of inoculations in human subjects which showed that in all probability pellagra was not infectious. At about the same time, Marzari (1817) suggested that maize might be deleterious because of its low nitrogen content and that the disease might be due to protein inanition. Early in the present century, the observation was made that one of the

important corn proteins, zein, was deficient in the amino acids tryptophan and lysine. About this time, studies of pellagra in Egypt led to the suggestion that the disease was related to lack of an essential amino acid, probably tryptophan.

Although there was evidence that the disease could be cured by changing the diet, not until 1918 was it associated with the absence of a dietary factor. In 1918 Goldberger et al claimed that pellagra included at least two aetiologically distinct syndromes and suggested that two dietary factors might be involved. Seven years later, Goldberger et al (1925) reported that pellagra could be cured by yeast and called the responsible factor the pellagra preventive factor.

In 1867 nicotinic acid which was subsequently shown to be pellagra preventive factor was prepared in pure chemical forms by Huber. It is produced with ease by oxidizing nicotine, thus by exerting the toxic effect of this alkaloid and leaving the water soluble B complex vitamin, nicotinic acid. In 1911 Funk, and later Suzuki, isolated nicotinic acid from rice polishings in their search for B_1. They observed that it had no beneficial effect in beriberi, and did not apparently suspect that they had actually isolated a vitamin capable of curing a different deficiency disease, pellagra. The discovery of nicotinamide as a component of coenzyme II (NADP) by Warburg and Christian in 1935 and a year later as a component of coenzyme I (NAD) by von Euler et al gave the original clue of the importance of nicotinic

acid in metabolism, and quickly thereafter led to the discovery of its role in preventing pellagra.

Elvehjem et al had been studying for years the effect of liver extracts on experimental blacktongue in dogs. Their effort finally culminated in the brilliant discovery in 1937 by Elvehjem et al that nicotinic acid would cure canine blacktongue and that nicotinamide could be isolated from the blacktongue - curative fraction of liver. Subsequent treatment of pellagrous humans with the compound added final confirmatory evidence (Spies et al, 1938).

Chemical Structure and Properties

Nicotinic acid (nicotinate) is a white crystalline solid which melts at 228°-229°C and is stable to light, heat, acids, and alkalies. It is sparingly soluble in cold water, but soluble in hot water and alcohol. It sublimes without decomposition on heating. It behaves as a base, forming a crystalline hydrochloride, and also as an acid, forming salts with metals, and esters with alcohol. It shows maximum absorption in the ultraviolet region at 262 nm. Its empirical formula is $C_6H_5NO_2$ and the molecular weight is 123.11.

Nicotinamide also known as niacinamide is pyridine-3-carboxylic acid amide. It melts at 128-131°C. It has a characteristic absorption curve in the ultraviolet region with maxima at 210, 220 and 260 nm. The absorption maxima of the nicotinamide is not, however, identical with that of the acid; the extinction at the maxima is usually less than with nicotinic acid; hence the sensitivity of most color

reaction towards nicotinamide is lower. Its empirical formula is $C_6H_6N_2O$ and its molecular weight, 122.13. It is hydrolysed to nicotinic acid by boiling with acid or alkali.

In natural products nicotinic acid occurs mainly in combined form (as coenzymes) and must be liberated by hydrolysis before assay. Chemical determination of the nicotinic acid and nicotinamide is based on the principle that pyridine derivatives give yellow colors with CNBr and a primary or secondary amine (Kutsky, 1973). Microbiological methods with Leuconostoc mesenteroides, however, appear to be more accurate despite its time-consuming and laborious work.

Nicotinic acid Nicotinamide

Figure 1. Chemical Structures of Niacin.

For biological activity, the pyridine molecule must have a substituent in the 3-position. Any change at positions 2, 4, 5 or 6 yields an inactive compound with the exception of quinolinic acid which readily decarboxylates to form nicotinic acid (Scott et al, 1976). Niacin functions metabolically as a component of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). The chemical structures of nicotinic acid and nicotinamide, are given in Fig. 1. above.

Occurrences

Nicotinic acid is widely distributed in grains and their by-products and in protein supplements. Liver, meat, poultry, peanut butter, and legumes are the richest sources. Milk and eggs, although low in preformed niacin, contain high amounts of tryptophan and as such have a high niacin equivalent. Free nicotinic acid apparently does not occur in the living organism but is found in the urine of animal. Nicotinic acid occurs in tissues in the form of its amide. Nicotinamide occurs to a much greater extent in the bound form than as the free nicotinamide. Thus, in rats, the bound nicotinamide occurs, in the liver, kidney, and muscle, while free nicotinic acid (or its amide) has been found only in the liver (Rosenberg, 1945).

Additional evidence now indicates the presence in many foodstuffs, especially cereals, of niacin-containing compounds from which the niacin is not nutritionally available. Much of the niacin in cereals such as rice and corn occurs as niacinogen, a peptide with a molecular weight of 12,000 to 13,000 and niacytin, a carbohydrate complex with a molecular weight of some 2,370. In these forms they are closely bound to protein and can only be separated by alkaline hydrolysis. For this reason the niacin in these foods has a low biological value and does little to meet the body's requirement for the vitamin unless prepared with alkali. Resolution of the important problems

of the relative activities of precursors and the biological availability of forms of niacin in foods deserves high priority.

Processing methods also influence niacin levels. For example, as much as 80% to 90% of the niacin of cereals is in the outer husk and is removed in the milling process (Darby et al, 1975).

Requirements

Recommended dietary requirements for nicotinic acid are influenced by two factors (Scott, 1976):
(1) nicotinic acid is synthesized in the animal body from tryptophan; thus the nicotinic acid requirement depends upon the tryptophan content of the diet and (2) much of the nicotinic acid present in many foods and feedstuffs is in a bound form that is not available to man and animals. It is released by treating the food materials with alkaline solutions. Other phenomena which may alter nicotinic acid requirements are the effects that various diets may have on the nicotinic acid synthesis by the gastrointestinal microflora. In view of this, it is very difficult to set the nicotinic acid requirement unless the tryptophan level is specified and it is known that the diet is adequate in pyridoxine, since this vitamin is needed in the synthesis of nicotinic acid from tryptophan. With growth as a criterion of adequacy of nicotinic acid intake, Hundley

(1949a) reported an average requirement for the rat of 5 mg per kg of food per day for diets containing 9% of casein plus 0.15% of cystine, 3% of gelatin, and 81% of starch. In an extensive experiment on pigs between 3 and 9 weeks of age, Powick et al (1947) reported the mean requirement for optimal growth lay between 15 and 25 mg of nicotinic acid per kg of niacin free casein. The requirement for nicotinic acid of the adult dog on the ration containing 19% casein and 66% sucrose are approximately 218 µg per kg of body weight (Schaefer et al 1942). Tappan et al (1952) have demonstrated that when the diet contained 9% casein and 82% of sucrose, 6-35 mg of nicotinic acid weekly or 1-4 gm of DL-tryptophan was adequate for optimum growth of the rhesus monkey. Adult ruminants on rations adequate for the normal growth of rumen microorganisms do not need a dietary supply of nicotinic acid (Hopper et al 1955). The horse, like the ruminant, apparently synthesized nicotinic acid in its digestive tract.

Almquist et al (1941) found the L-tryptophan requirement for optimum growth of the young chick to be approximately 0.5% of the diet. The results of the studies of Wilkening et al (1947) on the utilization of the isomers of tryptophan by chicks showed that from 17 to 40% D-tryptophan was utilized. Childs et al (1952) reported a requirement by chicks of 28 mg of the niacin per kg of diet, which contained approximately 28% of protein and 0.21% of tryptophan. They also indicated that excess tryptophan could

spare niacin to a slight extent but could not fully compensate for a partial niacin deficiency. It has been also suggested that chicken is dependent upon a dietary supply of niacin until at least 8 weeks of age. Fisher et al (1954) have reported that L-tryptophan could completely replace niacin, but that niacin did not spare tryptophan. They also pointed out that the minimum tryptophan requirement was a 0.15% L-tryptophan in the presence of adequate niacin. Sunde et al (1957) observed that the requirement of niacin for the young ringneck pheasant was 50 mg of total niacin/ kg of ration. According to experiments by Scott et al (1959) the niacin requirement was approximately 70 mg per kg of diet. From the results of experiments by Adams et al (1967), it is concluded that the niacin requirement for the laying hen for egg production and hatchability is less than 0.73 mg per day. The turkey poulet, according to Briggs (1946), requires 30-50 mg of nicotinic acid per kg of ration containing 18% of casein, 10% of gelatin, and 61% of glucose.

The Recommended Dietary Allowances (RDA, 1974) for human adults, expressed as niacin, is 6.6 mg per 1000 kcal, and not less than 13 mg at caloric intakes of less than 2000 kcal. The niacin allowance recommended for infants up to 6 months is 8 mg per 1000 kcal, and for children over 6 months and adolescents, 6.6 mg per 1000 kcal, but not less than 8 mg daily. The allowance recommended provides an increase of 2 mg of niacin daily during pregnancy, based on the recommended increase in energy intake. For lactation,

an additional daily allowance of 4 mg of niacin is recommended, consistent with the additional allowance of 500 kcal.

Hypervitaminosis

Nicotinic acid and nicotinamide are both quite non-toxic. The ratio between an effective therapeutic dose and a toxic dose is at least 1 to 1000. In rats and mice, the LD_{50}'s of nicotinic acid (Na salt when given parenterally) and nicotinamide range from 3.5 to 5.0 gm/kg subcutaneously and 5.0 to 7.0 gm orally. Nicotinamide is more toxic than nicotinic acid (Hundley, 1954). Inclusion of 1% of nicotinamide in a low-protein diet inhibited the growth of rats, whereas inclusion of 1% nicotinic acid in the same diet did not inhibit growth but induced fatty liver. Both effects could be prevented by methionine, the former also by choline plus homocystine, and the latter by choline and betaine. These and a number of other studies indicate that the toxicity of niacin and certain other pyridine derivatives may be explained partially, but not entirely, by depletion of the methyl groups of the body which are needed for excretion of these substances.

Much larger doses of nicotinic acid, but not nicotinamide, can cause a variety of pharmacologic responses. Massive doses of nicotinic acid were found to produce vascular dilation or flushing, with accompanying sensation

of burning or stinging of the face and hands. It has been used therapeutically in efforts to induce cerebrovascular dilation in senile ataxia, and as a harmless placebo in management of some hypochondriacs. Dosing with nicotinic acid of infants with kwashiorkor proved ineffective or injurious, probably because of the demand created by the added niacin for increased methylation in a state of protein deficiency with, often, pronounced hepatic injury. An extensively studied pharmacologic property of niacin is the lowering of serum cholesterol and lipoprotein concentrates by massive (usually 3 or more gms per day) oral doses of nicotinic acid. Nicotinamide does not share this effect. Among the metabolic effects of large doses of nicotinic acid two seem noteworthy: the decreased mobilization of fatty acids from adipose tissue in exercising subjects and the increased utilization of muscle glycogen stores. Long-term high-dose nicotinic acid therapy may in some instances be associated with appearance of laboratory evidence of diabetes and of hepatic injury, as well as activation of peptic ulcers all emphasize the non-physiologic levels of niacin involved. Intravenous injections of nicotinic acid in man result in the development of fibrinolytic activity which becomes apparent about 5 min after injection. Oral doses of nicotinic acid do not induce fibrinolysis even though they cause acute flushing and readily measurable blood levels of nicotinic acid. It is

hypothesized that the fibrinolytic response to nicotinic acid may be due to a rapid release of a stored activator (Weiner, 1979).

Biosynthesis

The many attempts to elucidate the niacin-tryptophan relationship have centered around two main ideas: (a) that bacteria in the gastrointestinal tract synthesize the vitamin and (b) that niacin synthesis from tryptophan takes place in the animal's tissues. The experimental evidence supporting the first hypothesis is as follows. The early observations concerning the marked influence of carbohydrates in promoting growth in rats receiving corn diets prompted Krehl et al (1946) to state that the effect of different carbohydrates on the growth depression might be explained on the grounds that they contribute to the establishment of an intestinal flora which is capable of synthesizing adequate amounts of the deficient factor. In a study on the intestinal synthesis of niacin and folic acid in the rat, Teply et al (1947) noted that dextrin diets caused the greatest synthesis of niacin as evidenced by analysis of the animal's cecal content. The role of the intestinal flora as an important source of niacin in the human has received strong support from the extensive studies of Ellinger et al (1945). The results of these workers indicate that the production and release of niacin by the

intestinal flora can amount to as much as 80% of the daily uptake. In a later report Ellinger et al (1949) observed that, although a mixed culture from rats' caecum was apparently able to produce nicotinamide from tryptophan, E. coli was not able to effect this transformation in the absence of lactate, whereas ornithine and, to a smaller extent, glutamine and arginine were readily converted into nicotinamide. Since more N'-methylnicotinamide was excreted when tryptophan was given to rats orally than when given parenterally, and since less was excreted when the animals were given sulfasuxidine, it was concluded that the nicotinamide was synthesized by the intestinal flora. It becomes evident that dietary circumstances which have a known influence on the character of the intestinal flora must be taken into consideration if an adequate appraisal of the nutritional status of an animal is to be made.

The contrary view supporting the second hypothesis is as follows. Symptoms of nicotinic acid deficiency in the pig, the dog, and in humans were relieved by the administration of L-tryptophan and the reserves of nicotinic acid and the urinary excretion of nicotinic acid metabolites were thereby increased (Rosen and Perlzweig, 1949). Administration of sulphasuxidine did not impair the action of tryptophan in relieving symptoms of pellagra in humans, although such a result could be anticipated if the intestinal flora were involved. Moreover, the intravenous administration of L-tryptophan to infants gave a prompt and large increase in

the urinary output of N'-methyl nicotinamide (Snyderman et al 1949). The synthesis of nicotinic acid by rats was apparently not affected by elimination of the bacterial flora or by enterectomy; and nicotinic acid was synthesized from tryptophan by rat liver slices (Hurt et al., 1949 ; Hundley, 1949b).

The tryptophan-niacin pathway, which is also known as the kynurenine pathway (Fig. 2), is important for production of nicotinic acid and provides also a means for degrading tryptophan to acetoacetyl-CoA, carbon dioxide, and ammonia. The amount of tryptophan metabolized by the various pathways available depends greatly on the amount of the amino acid in the diet. From the work with [^{14}C]-labelled tryptophan, Kallio et al (1949) and Mason et al (1951) reported that the liver of the normal rat is able to convert L-tryptophan into kynurenine, and formic acid was isolated from the livers. The first step was conversion to formylkynurenine which was then hydrolyzed by formylase. The studies with mutant strains of Neurospora crassa and with the rat have led to recognition of the fact that kynurenine (Beadle et al, 1947), 3-hydroxykynurenine (Mitchell et al, 1948), and 3-hydroxyanthranilic acid (Bonner, 1948) are intermediates in the conversion of tryptophan to niacin. Direct evidence for the metabolic relationship between tryptophan and niacin has come from isotope experiments in which labelled tryptophan or 3-hydroxyanthranilic acid was given to the rat and the

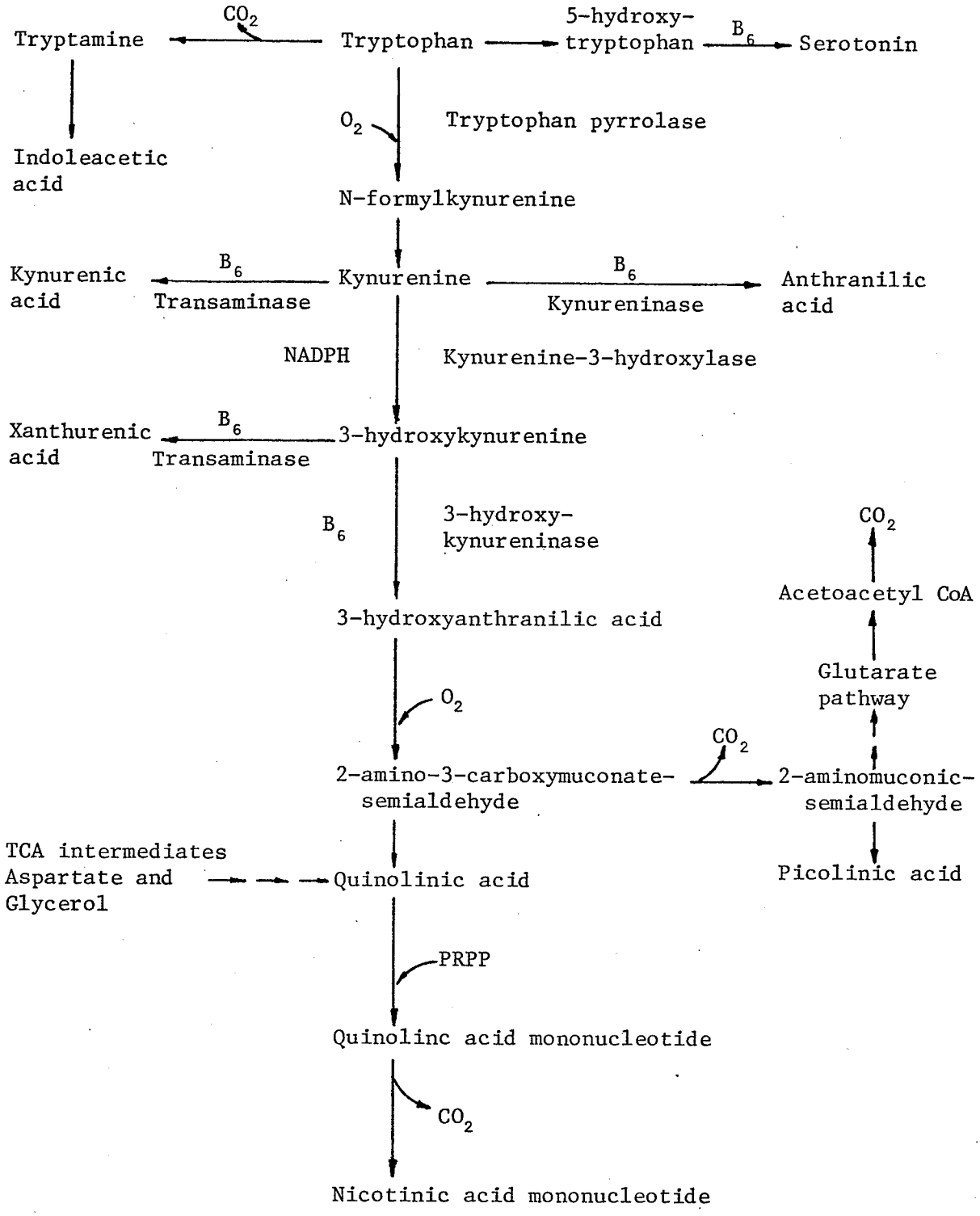


Figure 2. Pathways of tryptophan metabolism and nicotinic acid synthesis.

niacin recovered from the tissues and urine was analyzed. The results indicate that position 3 of the indole ring becomes the carboxyl carbon of niacin (Henderson et al, 1956) and that the nitrogen of the indole ring is converted to the pyridine ring nitrogen of niacin (Schayer et al, 1949). In 1951, Priest et al reported that a soluble enzyme system from liver and kidney catalyzes the conversion of 3-hydroxyanthranilic acid to quinolinic acid. Several investigators subsequently showed that the primary product of the oxygenation reaction was 2-amino-3-carboxy-muconate-semialdehyde and that cyclization of the latter resulted in the formation of quinolinic acid (Long et al, 1954). The latter reaction appeared to be a spontaneous reaction and did not require an enzyme. However, quinolinic acid has generally been considered to be a by-product of tryptophan metabolism rather than an intermediate in niacin biosynthesis since the compound is a very poor growth factor in the rat and Neurospora. In the several mammalian liver preparations studied by Mehler and May (1956), picolinic carboxylase was the only enzyme found to react with 2-amino-3-carboxymuconate-semialdehyde. The reaction produced picolinic acid and did not result in the formation of niacin. Nishizuka et al (1963) have recently found the conversion of quinolinic acid to nicotinic acid ribonucleotide in the presence of 5-phosphoribosyl-1-pyrophosphate by a soluble enzyme system obtained from the rat liver. Evidence is also presented which excludes free niacin as a dissociable

intermediate in this conversion. In higher plants and many microorganisms, quinolinic acid is formed from aspartate and glycerol (or closely related compounds) by a series of reactions which have not yet been elucidated.

Little of the tryptophan that enters the tryptophan-niacin pathway is actually used to form nicotinic acid ribonucleotide, and 60 mg of tryptophan results in the formation of only about 1 mg of nicotinic acid in humans. Recently Allen et al (1971) reported that in young growing chicks a conversion ratio of tryptophan to niacin is 45:1. Thus it would appear that this conversion ratio may not be constant and shows considerable variation depending upon the amount of tryptophan and preformed nicotinic acid available to the organism and also the amount of pyridoxal phosphate present. It has been reported that pyridoxine deficiency in animals can lead to increased excretion of xanthurenic acid and quinolinic acid in the urine after a tryptophan load (Kelsay, Miller and Linkswiler, 1968; Rose and Toseland, 1973). When pyridoxine is deficient, liver kynureninase located in the cytosol, becomes rapidly depleted of pyridoxal phosphate. However, the transaminases that metabolize kynurenine and 3-hydroxykynurenine to kynurenic acid and xanthurenic acid, respectively, are located in both kidney and liver and in both the cytosol and mitochondria. The mitochondrial transaminase becomes depleted of pyridoxal phosphate less rapidly. This accounts for the diversion of tryptophan metabolism to the formation of large amounts of xanthurenic acid in pyridoxine deficiency.

Not much is known about the factors that cause the elevation of quinolinic acid excretion due to the pyridoxine deficiency.

In fact quinolinate phosphoribosyltransferase which catalyzes the conversion of quinolinic acid to nicotinic acid mononucleotide has been shown not to be a pyridoxal phosphate-dependent enzyme (Gholson et al, 1964; Taguchi et al, 1974). Also, it has been suggested that tryptophan given in large doses may result in pyridoxine deficiency by combining with pyridoxal phosphate through the formation of Schiff bases (Hughes, 1966). It might be anticipated that pyridoxine deficiency would also result in deficient niacin synthesis. It is now generally accepted that the conversion of tryptophan to niacin does in fact take place in the tissues and that while synthesis by the bacterial flora of the intestine can and does occur, niacin from this source has little effect on growth (Robinson, 1966).

Absorption and Excretion

Nicotinic acid and nicotinamide are absorbed readily from the lumen of the intestine. There are no significant stores of niacin in the body. Following oral or parenteral administration of niacin, there is a transient rise in the blood niacin level, and the metabolites are excreted in the urine (Svedmyr et al, 1970).

Loss of nicotinyl structure from the pyridine nucleotide cycle may occur at the level of nicotinamide or

nicotinate, which are excreted either as derivatives or as the free compounds depending on the species concerned. Huff and Perlzweig (1941) reported that rats were able to convert a substantial proportion of ingested nicotinate into trigonelline and nicotinuric acid, only a small amount being excreted unchanged. The formation of nicotinuric acid from nicotinate has also been reported in the dog, hamster and mouse (Leifer et al, 1951) as well as in the rabbit, tortoise and frog. A study of the synthesis of nicotinuric acid in rat kidney slices and homogenates demonstrated that it was a two-stage process (reactions 1 and 2) involving nicotinyl-CoA (Jones, 1959).

$$\text{Nicotinate} + \text{ATP} + \text{CoASH} \longrightarrow \text{nicotinyl-CoA} + P_i + \text{ADP} \qquad (1)$$

$$\text{Nicotinyl-CoA} + \text{glycine} \longrightarrow \text{nicotinuric acid} + \text{CoASH} \qquad (2)$$

Dinicotino-ornithine is the major excretory metabolite of nicotinate in chickens (Dann and Huff, 1947); it is probably formed in a similar fashion to nicotinuric acid but no coenzyme requirement has been reported. Koeppe and Hall (1956) pointed out that nicotinuric acid constituted about 60% of the tertiary nitrogen derivatives of nicotinate excreted by normal rats, but it formed a much lower proportion in the case of pantothenic acid-deficient rats. This result suggests that coenzyme A is involved in the biosynthesis of nicotinuric acid as in the enzymic conversion of benzoic acid into hippuric acid. Somewhat similar results were obtained with dogs, which apparently excreted trigonelline

and nicotinuric acid as the principal end-products of nicotinate metabolism (Sarett, 1942).

The excretion of another metabolite of nicotinate was first noted by Najjar and his co-workers (Najjar and Holt, 1941). They observed that pellagrins excreted in the urine a fluorescent substance designated F_1, which disappeared when the patients were treated with nicotinate, and was replaced by another fluorescent compound known as F_2. Later F_2 was shown to be N'-methylnicotinamide which is a characteristic urinary excretion product of nicotinate in animals. Cantoni (1953) demonstrated that N'-methylnicotinamide is formed only in liver under the influence of nicotinamide methyltransferase (S-adenosylmethionine: nicotinamide methyltransferase) with S-adenosylmethionine as methyl donor (reaction 3).

nicotinamide + S-adenosylmethionine $\longrightarrow$ N-methylnicotinamide + S-adenosylhomocysteine (3)

Kaplan (1961) provided an evidence that N'-methylnicotinamide may come from the nicotinamide resulting from the breakdown of NAD in liver. Nicotinate is not methylated by nicotinamide methyltransferase, which accounts for the absence of the betaine and trigonelline from animal tissues. In rats nicotinamide added at a level of 1% to the diet inhibited growth; nicotinate had no such effect. However, they both produced "fatty livers", a characteristic symptom of a deficiency of available labile methyl groups. Presumably the nicotinamide methyltransferase activity is so

great that when high levels of nicotinamide reach the liver they trap all the available methyl groups and thus produce a methyl deficiency syndrome. Nicotinate is less effective than nicotinamide probably because it has first to be metabolized to nicotinamide, via NAD (Handler and Dann, 1942).

Using the technique of paper chromatography Reddi and Kodicek (1953) showed that the main metabolites excreted by humans following a 100 mg dose of nicotinate are, in decreasing order, nicotinuric acid, N'-methyl-nicotinamide and nicotinamide. On the other hand, Ginoulhiac et al (1962) indicated that 6-pyridone of N'-methylnicotinamide is the principal urinary excretion product resulting from the oxidation of N'-methylnicotinamide.

Deficiency

A) General

In their earlier studies on the chick antidermatitis factor, Mickelsen et al (1938) attempted to produce a nicotinic acid deficiency in chicks through the use of the modified Goldberger diet. They found that chicks on this ration grew very poorly but showed no external symptoms. Helmer et al (1938) demonstrated that the Goldberger diet (1926) was deficient in thiamine and riboflavin as well as in nicotinic acid.

Briggs et al (1942), using purified rations low in nicotinic acid, conducted the investigation of the role of nicotinic acid in chick nutrition. In the absence of nicotinic acid there was a depression in the growth rate and the appearance of a deficiency condition similar to canine blacktongue. A decrease in food consumption was also noticed. Upon the addition of nicotinic acid to the basal ration, food consumption and growth increased and the chick blacktongue symptoms disappeared within a few days. However, Dann and Handler (1941) and Snell and Quarles (1941) have presented evidence for the occurrence of a marked increase in the nicotinic acid content of the incubating egg and suggested, therefore, that the hatched chick does not require a dietary source of this vitamin. However, Briggs et al (1942) indicated that if such synthesis of nicotinic acid does exist in the growing chick there is not sufficient formation to supply enough of the vitamin for the optimal growth and for the prevention of chick blacktongue. On the basis of this observation they concluded that the growing chick requires a dietary source of nicotinic acid for optimal growth. In addition, they also found nicotinamide equally effective in stimulating growth and preventing blacktongue.

Krehl et al (1945) have reported that rats fed a 9% protein diet containing 40% corn developed a growth depression which was corrected by either niacin or tryptophan.

In work with dogs Krehl et al (1945) produced a nicotinic acid deficiency by the addition of corn grits to a synthetic ration. In work with swine, Davis et al (1940) noted necrotic enteritis, due to a deficiency of nicotinic acid, especially prevalent in pigs fed diets rich in corn in contrast with other grains. Briggs et al (1945) reported that the addition of 10% of gelatin to a highly purified ration caused an increase in the nicotinic acid requirement of chicks and that tryptophan acted similarly to nicotinic acid in preventing the deficiency. Arginine, glycine, and alanine have been known to account for some of the depressing action of gelatin. It was speculated that nicotinic acid is necessary for the metabolism of excess NH_2 groups. To further emphasize the importance of amount and character of dietary protein, especially with regard to tryptophan content, Krehl et al (1946) showed that egg albumin, fibrin, and soybean globulin at dietary levels of either 10 or 15% protected the rat from the usual growth inhibition which resulted when corn grits were added to the diet. This is in marked contrast to results obtained with casein at comparable levels and is undoubtedly dependent upon the higher tryptophan content of these proteins, as compared with casein. The specificity of tryptophan in this relationship is indicated at least by the fact that addition of lysine, arginine, methionine, and histidine to the corn supplemented ration at levels of 0.5% failed to elicit a growth response. The importance of dietary protein and tryptophan in the nutrition of dogs on a niacin deficient

diet has been reported by Singal et al. (1948a). They noticed that niacin deficiency in dogs was not prevented or significantly altered by the inclusion of the tryptophan-deficient proteins, zein, or gelatin, in the diet.

B) Amino acid imbalance

Although previous experiments with wheat gluten and gelatin indicated that an undesirable growth rate might result when niacin and tryptophan are simultaneously low, Krehl et al (1946) looked at the effect of a niacin-free diet containing 9% casein and 6% gelatin along with different carbohydrates on the growth of rats. In all cases growth inhibition was produced by the addition of gelatin in much the same way as when wheat gluten had been used instead of casein. Since this is the same condition that prevails in corn-supplemented rations, the effect of zein, the principal protein in corn, was tested by adding it at a level of 3% to a 9% casein ration at the expense of carbohydrate. This addition of zein resulted in very poor growth which was correctable by the addition of tryptophan or niacin.

As a result of such investigations, Krehl et al. (1946) proposed that the action of corn in niacin-low synthetic rations is a function of the character of the predominant protein present, zein, and this in turn is due to an amino acid imbalance in this protein. It was further stated that an

imbalance of amino acids results in an increased tryptophan or niacin requirement, and therefore poor growth, is evident by the fact that rats will grow reasonably well on rations which contain the same amount of tryptophan and which also contain a well balanced protein. They also indicated that this imbalance of amino acids could be extended beyond proteins such as gelatin and zein, when it was noted that the addition of acid-hydrolyzed proteins such as fibrin, egg albumin, and casein at levels as low as 2% inhibited growth. Since such findings suggested the possible involvement of a specific amino acid, glycine, leucine, and glutamic acid were added separately and collectively at a level of 1% to 9% casein-dextrin ration. No growth inhibition was observed, but when sucrose was used as the carbohydrate in place of dextrin, the addition of 2% glycine and a combination of L-tyrosine, DL-phenylalanine, and DL-valine along with glycine and L-leucine resulted in a retarded growth rate which was markedly stimulated by the addition of niacin. Groschke et al (1946) also observed that the addition of certain pure amino acids alone and in combination to synthetic diets low in niacin produced growth inhibition in chicks analogous to that noted with gelatin. Niacin in all cases overcame this inhibition. Glycine alone, arginine and glycine together, and especially arginine, glycine, and alanine in combination showed marked inhibitory action. These authors suggest that niacin is concerned in some manner with the metabolism of amino acids

especially glycine, arginine, and alanine.

In a further study on growing chicks and on laying hens, Briggs et al.(1946a) reported that niacin deficiency may occur in chickens under practical conditions if the protein (amino acid) content is unbalanced and that the niacin requirement of chickens is influenced by the protein content of the ration and in particular by the presence of excessive amounts of proteins such as gelatin which are deficient in tryptophan. Groschke et al (1947) also pointed out that the chick-pellagra symptoms caused by feeding zein are due to the accumulated action of the amino acid constituents alone in this protein. Of the amino acids in zein, glutamic acid, leucine, alanine, proline and phenylalanine seemed primarily concerned. An indication of nonspecificity of amino acids in this mechanism was indicated by the above mentioned work with rats and chicks and was further demonstrated by the report of Groschke et al (1948) when they found that at a level of 4% in the diet, nearly all of 17 amino acids tested depressed the growth rate to some extent. DL-methionine proved particularly effective in this respect. Another study on the amino acid imbalance affecting the growth of rats on a niacin-tryptophan deficient diet was reported by Henderson et al (1947). Growth retardation was observed in rats when the 9% casein-sucrose, niacin-free diet was supplemented with 2% glycine, 2% acid hydrolyzed casein, or an amount of crystalline amino acids equivalent to 2% acid hydrolyzed casein. Again the

growth depressing effect of glycine could not be shown on a diet which contained dextrins as the carbohydrate .

Singal et al (1947) working along a similar line with rats noted that the addition of histidine, valine, threonine, and lysine, to the low protein (9% casein) diet produces a marked growth depression which is not accompanied by a decrease in liver niacin. Hankes et al (1948) were able to demonstrate that the specific amino acids which most effectively induced a niacin-tryptophan deficiency in the rat were DL-threonine and DL-phenylalanine in amounts equivalent to that present in 2% acid hydrolyzed casein, i.e., 0.078% and 0.104% respectively. DL-phenylalanine was not as effective as DL-threonine and furthermore the growth inhibition with these amino acids was not marked when dextrin was used instead of sucrose as the carbohydrate. Singal et al. (1948b) confirmed the above observation concerning the growth retarding action of added dietary threonine for the rat and further noted that the growth inhibition is not accompanied by a decrease in the storage of niacin in the liver or muscle.

Hankes et al (1949) reported that tryptophan is spared for conversion to niacin when the rate of protein synthesis is limited by some other amino acids, such as threonine. However, upon the addition of threonine to the diet, tryptophan assumes the role of the most limiting amino acid and is drawn into protein at the expense of niacin formation, thus causing a deficiency of this vitamin.

Henderson et al.(1953a,1953b) induced niacin deficiency in rats by raising the levels of threonine in diets containing marginal levels of tryptophan. Growth was severely suppressed when threonine was increased from 0.33 to 0.38% of a diet that contained 10% hydrolyzed casein and 0.1% DL-tryptophan. They also suggested that the age and size of the animal was of importance in producing tryptophan-niacin deficiency.

Further investigations were undertaken to see whether excess leucine would also bring about such disturbances in the tryptophan-nicotinic acid interrelationship. In 1954 Harper et al discovered that amino acid imbalance, created by the addition of 3% leucine to 9% casein diet, could be largely corrected by the simultaneous addition of 1.0% isoleucine. In view of the structural similarity of the two molecules, they postulated an antagonism between the two amino acids, whereby leucine prevents the normal utilization of isoleucine. Amino acid imbalance due to an excess of leucine in jowar has also been suggested as a possible factor in the development of pellagra which is endemic in certain population groups that subsist principally on this millet (Gopalan et al 1960). In support of this hypothesis, it was further demonstrated that the oral administration of leucine to normal human subjects brought about a significant increase in the excretion of quinolinic acid and a significant decrease in the excretion of 6-pyridone of N'-methylnicotinamide in the urine. There was

also a significant decrease in the excretion of both 5-hydroxyindoleacetic acid and free indoleacetic acid. In the presence of a tryptophan load, these effects of leucine administration were more pronounced. To ensure that these effects were specific for leucine, the effect of oral administration of lysine to a group of subjects was investigated by Belavady et al (1963). This study demonstrated that lysine administration had no effect on the urinary excretion of tryptophan metabolites. In line with these observations on human subjects, Raghuramulu et al (1965a) reported that urinary excretion of quinolinic acid and N'-methylnicotinamide were increased in both young and adult rats when leucine was added at 1.5% level to a 9% casein diet. Quinolinic acid excretion was more markedly affected in young rats, whereas N'-methylnicotinamide excretion was more affected in adult rats. Isoleucine counteracted the effect of leucine in young rats. In order to demonstrate the possible role of leucine in the pathogenesis of pellagra they induced a classical black tongue in dogs by feeding jowar, which is not low in tryptophan but contains considerable amounts of nicotinic acid. In 1966 they also investigated the nature of nicotinic acid present in the millet jowar to determine if poor availability of nicotinic acid in jowar may be partly responsible for the occurrence of pellagra in jowar-eaters (Belavady et al). Growth of rats and pups fed jowar or lime-treated jowar diets revealed that animals consuming untreated jowar grew better, giving the inference

that nicotinic acid in jowar is, in fact, in the available form. Their next studies were directed towards elucidating the precise biochemical mechanisms underlying the effect of leucine on nicotinic acid metabolism. In order to establish this effect, nicotinamide nucleotide synthesis *in vitro* in the erythrocytes was studied in normal subjects as well as in patients suffering from pellagra before and after the administration of leucine supplements. They observed that the total nucleotide concentration in erythrocytes of pellagrins was not lower than that in normal subjects, but the ability of the erythrocytes to synthesize these nucleotides *in vitro* was significantly lower. Oral administration of 10 g leucine daily, for 5 days, depressed the nicotinamide nucleotide synthesizing ability of erythrocytes both in normal subjects and pellagrins. This, however, was not accompanied by changes in the actual nucleotide concentration in the erythrocytes themselves. These apparently paradoxical phenomena would suggest that distribution of different nucleotide fractions of erythrocytes in pellagrins may be different from that of normal subjects.

In 1973, Ghafoorunissa *et al.* reported that administration of leucine at 3% level in a 9% casein diet to young rats resulted in a significant increase in the activity of tryptophan pyrrolase in liver and a significant decrease in the activity of quinolinate phosphoribosyltransferase in the liver and kidney. In addition, a significant increase in the activity of picolinate carboxylase, a key enzyme in the

oxidation of tryptophan to CO_2 through glutarate pathway, was observed. In contrast to Gopalan's reports, Truswell et al (1963) failed to see the anticipated elevation in urinary N'-methylnicotinamide by the excess intake of leucine. Recently Nakagawa et al (1975) reported on their first studies of Gopalan's hypothesis. They found that there were no consistent trends in the amount of excretion of N-methylnicotinamide or nicotinic acid during the high leucine period. In their second experiment, 5 female subjects were fed the basal diet for 6 days at which time the leucine intake was elevated to 11 g. Pyridone excretion was significantly reduced in all subjects. Excretion of quinolinic acid, 5-hydroxyindole acetic acid, and nicotinic acid was constant throughout the control and experimental period in all 5 subjects. In their 3rd experiment, they examined the effect of leucine supplements (11 g per day) in 3 female subjects when pyridoxine was omitted for the entire 20 day test period. Once again, pyridone excretion declined in 2 of 3 subjects and urinary nicotinic acid was constant but a trend toward an elevation of 5-hydroxyindole acetic acid and N'-methylnicotinamide and a decline in quinolinic acid was noted. From these observations they concluded that their data offered no support for the contention that an excess intake of leucine causes changes in the excretion of tryptophan and niacin metabolites.

Recently Krishnaswamy et al (1976) observed that in normal human volunteers, simultaneous administration of

pyridoxine orally with leucine could counteract some of the metabolic effects of excess leucine, i.e. increased quinolinic acid excretion, decreased *in vitro* nicotinamide nucleotide synthesis in erythrocytes and abnormalities in 5-hydroxytryptamine metabolism. The results showed that 30 g leucine/ kg diet significantly reduced the quinolinate phosphoribosyltransferase when the diets provided 5 µg pyridoxine/10 g and that the effect was only marginal when the diet included 30 µg pyridoxine/10 g. The inhibitory effect was completely absent when the diet provided higher amounts of pyridoxine (60 µg/10 g). In a later study (1978) they also reported that dietary excess of leucine induces leucine aminotransferase which requires pyridoxal phosphate as coenzyme, thus modifying the requirements for vitamin B_6. It may, therefore, be expected that the enhanced activity of leucine aminotransferase may cause the increased catabolism of leucine. These results suggest that in endemic pellagra arising as a result of high intake of leucine from jowar, the vitamin B_6 content of the diet may be an important determinant of the extent of impairment of the tryptophan-niacin pathway. According to Bapurao (1975) the vitamin B_6 content of jowar is only half that of wheat and less than that of rice.

C) Antimicrobial agents

An antimicrobial agent is so called because it has the capacity to inhibit the growth of, to alter, or to destroy bacteria in vitro or in vivo (1). The spectrum of antimicrobial activity can vary from destruction of bacteria to the production of slight changes in structure or metabolism. Marshall et al (1940) reported that sulfaguanidine appears to be the most effective bacteriostatic agent for various intestinal bacteria. Black et al (1947) attempted to find out whether intestinal bacteria may synthesize certain unidentified factors essential in the nutrition of the rat. They observed that the growth of rats was reduced from the average of 25 gm per week to less than 10 gm per week when 0.5% of sulfaguanidine was added to their synthetic diet containing 18% of purified casein and 76% of sucrose. It has been suggested that the reduced growth effect is attributed to the inhibition of intestinal synthesis by responsible microorganisms (Fig. 3).

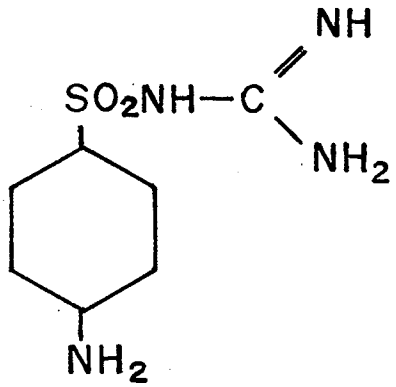


Figure 3. Chemical Structure of Sulfaguanidine.

In 1951, Coates et al. reported that penicillin supplementation accentuated the deficiency of niacin when the chicks were fed a purified diet with dextrin as the carbohydrate. In contrast, Biley et al (1952) have presented evidence that aureomycin spares dietary niacin. However, Nelson et al (1953) resolved these contrasting observations reported by Coates et al and Biley et al. They demonstrated that niacin deficiency symptoms were neither accentuated nor alleviated by supplementing a semipurified diet (a casein-gelatin) with aureomycin or procaine penicillin. Neither aureomycin nor penicillin stimulated chick growth when the diet was severely low in niacin (0.48 mg/kg).

D) Antiniacin

General

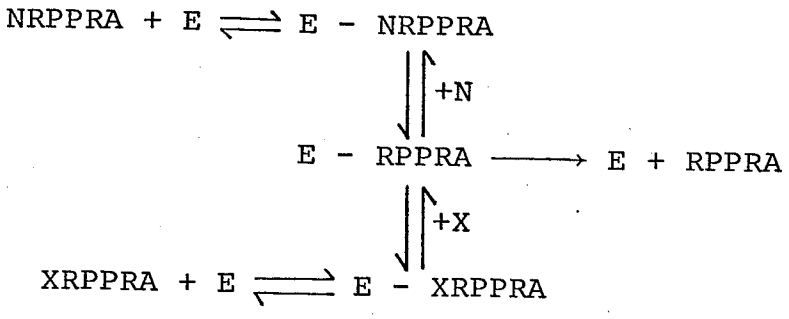
Most coenzymes are derivatives of vitamins and it has been anticipated that analogs would induce vitamin-deficiency states. This has been demonstrated in some cases; that is, effective analogs have been found to produce a pattern of symptoms roughly similar to those seen in deficiency of the corresponding vitamin. Nevertheless, it should be clearly understood that the situations are basically different. A dietary restriction of a vitamin leading to a generalized depletion in the tissues would not necessarily bring about functional changes identical to those caused by an analog, which could be much more effective in interfering with

certain functions of the coenzyme than simple depletion and possibly leave other functions untouched. Even though the analog primarily interfered with the transport of the vitamin into the cell, or blocked its further metabolism to the active coenzyme, it is not justifiable to conclude that a state of generalized depletion will result, because these effects will presumably not be exerted equally on all tissues. The differential penetration of the analog into the various tissues will perhaps be one important factor in determining the response.

Contrary to vitamin depletion, analogs often cause a rise in the renal excretion of coenzyme or its metabolites, due to the displacement of the normal coenzyme by the analog in the tissues and its release from the cells. The analog might also alter the formation of the coenzyme from its precursors, or inhibit the metabolism of the active coenzyme, or in some manner change the renal excretion of resorption of the coenzyme or its precursors, so that a variety of effects on over-all excretion is possible. Furthermore, in many instances the analogs are metabolized along the same pathways as the coenzymes to form inhibitory products. The direct effect of a coenzyme analog on the enzyme reaction requiring the cooperation of the coenzyme will depend on the tightness with which the coenzyme is bound to the enzyme. Some coenzymes are so tightly bound so that they remain on the enzyme through numerous isolation procedures, and in such cases the addition of an analog, even though it has a high affinity for the enzyme, may not be able to

replace the natural coenzyme rapidly enough to induce inhibition. It must be remembered that the analog does not actively displace the coenzyme (i.e., it does not force it from the enzyme) but only binds to the free enzyme; if essentially all of the enzyme is combined with coenzyme, there is little opportunity for the analog to act. It has been frequently stated that coenzyme analogs are specific inhibitors. This is true in one sense inasmuch as these analogs or their metabolic derivatives appear to interfere only with those enzymes or reactions involving the corresponding normal coenzymes, in most instances. On the other hand, the coenzymes often participate in several different types of metabolic activity so that the metabolic disturbances produced by the analogs may not be specific with respect to a single reaction (Webb, 1966).

It has been reported that NADase can catalyze an exchange reaction between the nicotinamide moiety of NAD and compounds related to nicotinamide according to the following equation (Kaplan et al, 1954):

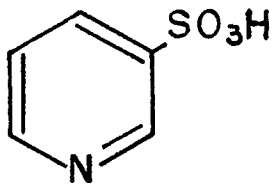


where NRPPRA is NAD, E is NADase, XRPPRA is the NAD analog,

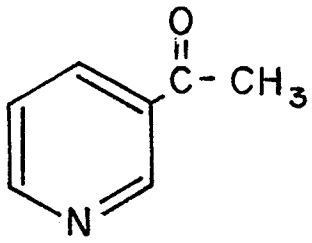
E-RPPRA is the relatively stable ribosyl enzyme complex, and X is the pyridine derivatives exchangeable with nicotinamide. The overall exchange reaction would be:

$$NRPPRA + X \rightleftharpoons XRPPRA + N$$

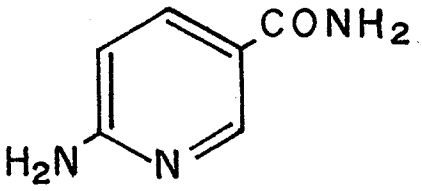
These NADase which catalyze the hydrolysis of NAD at the nicotinamide-ribose linkage might be considered as trans-glycosidases and able to transfer the RPPRA group to compounds structurally related to nicotinamide to form NAD analogs (Zatman et al. 1954). The structures of some antiniacins are shown in Fig. 4.



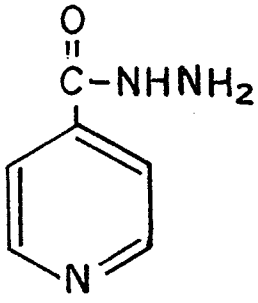
pyridine-3-sulfonic acid



3-acetylpyridine



6-aminonicotinamide



isonicotinic acid hydrazine

Figure 4. Chemical Structures of Antiniacin.

(1) 3-Acetylpyridine

In a search for pyridine derivatives which might have vitamin activity against black tongue in dogs, Woolley et al (1938) observed that 3-acetylpyridine is not only ineffective but kills nicotinamide-deficient animals in 1 day, normal dogs being unaffected. In 1945 Woolley induced a niacin deficiency in mice and animals succumb at the LD_{50} (around 3 mg 3-acetylpyridine per day) in 3 to 4 days. However, mice can be completely protected by providing nicotinic acid or nicotinamide in the diet. On the other hand, yeast and most bacteria seem to be quite resistant to 3-acetylpyridine, although the growth of Lactobacillus casei in nicotinate-free medium is inhibited around 50% at 16.5 mM, a depression that can be reversed by nicotinate but not by nicotinamide. These early observations all point to the interference by 3-acetylpyridine in the metabolism or function of nicotinic acid or nicotinamide. If it is assumed that the primary role of these metabolites is the formation of the NAD and NADP coenzymes, the following possible mechanisms for inhibition by 3-acetylpyridine might be imagined. (1) inhibition of some step in the synthesis of NAD, (2) inhibition of the interconversion of nicotinate and nicotinamide, (3) entrance into one of the pathways of nicotinate metabolism to form inhibitory intermediates, (4) formation of an NAD analog (3-acetylpyridine adenine dinucleotide), either through the normal pathway or by the exchange reaction catalyzed by NADase,

and (5) direct interference with NAD or NADP to inhibit dehydrogenase activity.

In an attempt to solve the problem of how 3-acetylpyridine induces its inhibitory effects, Gaebler et al (1951) found that 3-acetylpyridine at doses around 0.5 g/day increases the urinary excretion of N'-methylnicotinamide in both normal and nicotinic acid deficient dogs. They also indicated that the N'-methylnicotinamide could arise either from a disturbance of nicotinamide metabolism, since N-methylation is an important reaction in the elimination of nicotinamide, or directly from the 3-acetylpyridine. In a later study they pointed out that N'-methylnicotinamide is formed from 3-acetylpyridine and this analog might also interfere with nicotinic acid metabolism in the course of its oxidation and methylation (Beher et al, 1952). They also made a very interesting speculation that the oxidation of 3-acetylpyridine may involve NAD(P) enzymes; this might mean that in nicotinic acid deficient animals, where NAD(P) levels are low, the oxidation of 3-acetylpyridine would be impaired and the analog would be more toxic.

According to McDaniel et al (1955) 3-acetylpyridine at low dosage (25-60 mg/day) can protect against black tongue in dogs but at higher dosage it can create a nicotinic acid deficiency. Thus it has been suggested that animals may have a limited ability to oxidize 3-acetylpyridine; small amounts are mainly oxidized to normal metabolites and little 3-acetylpyridine is left to inhibit,

whereas the larger doses exceed the metabolic capacity of the system. This is indirectly supported by the results of Guggenheim et al (1958), who determined the excretion of N'-methylnicotinamide in rats given comparable doses of nicotinamide and 3-acetylpyridine. They observed that beyond a dose of 50 mg/kg there seems to be relatively less oxidation of the analog. Further evidence confirming the conversion of acetylpyridine to nicotinamide was presented by Kaplan et al (1954). They found that administration of 3-acetylpyridine (500 mg/kg of body weight) to mice leads to a four-fold rise in the NAD level of the liver as compared to the control. Despite a great increase in NAD level, no 3-acetylpyridine analog of NAD was detected in the liver. In view of these facts observed, it was concluded that the metabolism of 3-acetylpyridine and the compounds derived from it thus depend on the species, the dose, and whether the animals are normal or nicotinic acid deficient.

It was shown that 3-acetylpyridine-NAD can replace NAD as the coenzyme for the various dehydrogenases. In some cases 3-acetylpyridine-NAD can be reduced more rapidly than NAD (horse liver alcohol dehydrogenase, beef liver glutamate dehydrogenase, Lactobacillus D-and L-LDH) and in other cases proceeds more slowly (yeast ADH, beef heart LDH, yeast glyceraldehyde-3-p-dehydrogenase), while in a few instances the rates are approximately equivalent (rabbit muscle LDH) (Kaplan et al; 1956; van Eys et al, 1958; Kaplan, 1959; Stockell, 1959). 3-Acetylpyridine-NADP is

reduced about one-fifth as fast as NADP in the pig heart IDH (isocitrate dH) system and is inactive in erythrocyte glucose-6-P dehydrogenase (Marks et al, 1961). It was also demonstrated that the relative rates do not necessarily reflect the relative bindings to the dehydrogenases. The introduction of 3-acetylpyridine may bring about an imbalance of the normal relative substrate oxidation due to the alteration of the rates of the various dehydrogenases in different ways.

(2) Pyridine-3-sulfonate and pyridine-3-sulfonamide

These analogs might be expected to exert some inhibitory actions on nicotinic acid and nicotinamide metabolism (Webb, 1966).

McIlwain (1940) found that in the absence of nicotinic acid or nicotinamide the pyridine-3-sulfonate and pyridine-3-sulfonamide inhibit the growth of various bacteria and the inhibitions can be overcome by nicotinic acid and nicotinamide. In 1942 von Euler reported that pyridine-3-sulfonic acid inhibited LDH when the concentration of the coenzyme was kept constant and that of the analog was increased, so that the latter would appear to compete with the coenzyme for the apoenzyme. However, the inhibitory action decreased as the concentration of the coenzyme or of nicotinic acid or nicotinamide was increased. He also demonstrated that the affinity of NAD for apodehydrogenase was 2 to 3 times that of pyridine-3-sulfonic acid, while nicotinic acid,

benzoic acid and benzene sulfonic acid had about the same affinity as pyridine-3-sulfonic acid for apodehydrogenase. It was concluded that the inhibition of enzyme activity by these compounds was not due soley to the carboxylic acid or sulfonic acid groups but was rather a function of the whole molecule. According to Karrer et al. (1946), the antagonistic action of pyridine-3-sulfonamide towards nicotinamide was probably not due to displacement of the latter by the former from NAD or NADP. If, however, displacement actually does occur, then the altered NAD is probably capable of reversible reduction, since pyridine-3-sulfonamide methiodide and ethiodide were reduced by sodium dithionite to 1-methyl-and 1-ethyl-1,2-dihydropyridine-3-sulfonamide, respectively. Woolley et al. (1943) observed that pyridine-3-sulfonic acid did not produce symptoms of nicotinic acid deficiency when fed to mice at 5% in the diet, nor did it affect the excretion of N'-methylnicotinamide by normal or nicotinic acid deficient dogs, the coenzyme content of the erythrocytes or the health of the animal. In addition, Hicks (1955) observed hippocampal necrosis in only one animal given pyridine-3-sulfonic acid, so that it is presumably not so effective as 3-acetyl-pyridine. It has also been presented that brain NADase is not inhibited by pyridine-3-sulfonamide (McIlwain, 1950), and the sulfonate does not significantly inhibit either beef spleen NADase (Zatman et al., (1954) or nicotinamide deamidase (Grossowicz et al, 1956).

(3) Isonicotinic acid hydrazine (INH)

To explain the potent antitubercular activity of isonicotinic acid hydrazine, Zatman et al (1954) considered the possibility that INH may exchange with the nicotinamide moiety of NAD to yield a toxic NAD analogue. The analogue (INH-AD) has been isolated from an incubation mixture containing NAD, INH, and NADase from pig brain and from beef spleen (Goldman, 1954). However, it does not inhibit NAD-requiring dehydrogenase reactions. Although the occurrence has been reported of INH-induced pellagra in tuberculosis patients which completely responded to nicotinamide treatment, there is no evidence to indicate that the deficiency symptoms were due to the formation of INH-AD. More recent investigations by Gangadharam et al (1963) also suggests that INH exerts its bactericidal action by selectively inhibiting nucleic acid synthesis.

(D) 6-Aminonicotinamide (6-AN)

6-AN is a structural analogue of nicotinamide, differing from it only by the presence of the amino group on carbon 6. This analogue has been suggested to be the most potent nicotinamide antagonist available (Johnson et al, 1955). The acute LD_{50} in mice is 35 mg/kg, although 2 mg/kg/day leads to 50% mortality by the 11th day. His subsequent studies demonstrated that the lethal toxicity of 6-AN could be completely blocked by concurrent administration of nicotinamide, nicotinic acid, or tryptophan

(Johnson et al, 1956). According to Halliday et al (1957), it is very toxic to rabbits, producing loss of motor control and paralysis, and in rats it produces these and other signs of nicotinic acid deficiency. The endogenous respiration of liver homogenates from 6-AN treated mice is depressed 70% and lactate oxidation is depressed 49%; addition of NAD counteracts these depressions. However, no effect is observed when the analogue is added directly to liver slices. The delayed action of 6-AN when injected into animals suggested that 6-AN activity was contingent upon metabolic transformation in vivo, probably by incorporation into pyridine nucleotides in place of nicotinamide to produce the corresponding 6-AN analogue (Kaplan et al, 1954). A substance was isolated from the tissues of rats and mice following incubation of NAD, 6-AN, and NADase, which proved to be the 6-AN analogue of NAD. This analogue is completely inactive with yeast alcohol dehydrogenase. Formation of 6-AN adenine dinucleotide in vitro and in vivo was confirmed by Dietrich et al.(1958b), who also demonstrated that the corresponding 6-AN analogue to NADP was formed in vivo. These analogues underwent none of the addition reactions typical of natural pyridine nucleotides. However, it has been postulated that these impotent pyridine nucleotides become bound, in vivo, to apo-dehydrogenases, producing inactive holoenzymes. Dietrich et al.(1958a) have studied the effect of 6-AN administration on the activities of several NAD-linked enzyme systems in the 755 adenocarcinoma trans-

planted into C57BL mice. LDH was unaffected, while 3-phosphoglyceraldehyde dehydrogenase, β-hydroxybutyrate dehydrogenase, and α-ketoglutarate oxidase activities were markedly inhibited. In the 755 adenocarcinoma, where a relatively low enzymatic capacity to carry out oxidative phosphorylation is coupled with a high energy requirement, (indicated by the very rapid growth of the tumor), marked lowering of the ATP and ADP, and increases in the AMP concentration were observed upon the administration of 6-AN. Thus, it would appear that 6-AN owes its biological activity to the formation of the corresponding analogues of NAD and NADP, which are unable to function as normal hydrogen carriers, but compete with the normal co-enzymes for active sites on the enzyme. Consequently, they postulated that interference with pyridine nucleotide metabolism in this manner would be expected to exert a pronounced effect on the over-all metabolism of tissues, since most metabolic reactions are directly or ultimately NAD- or NADP-dependent.

As previously stated by Halliday et al (1957) 6-AN exerts a depressing action against the growth of certain lymphosarcomas and adenocarcinomas, and this is reversed by nicotinamide. Tumor regression occurs at 3-4 mg/kg/day but some weight loss also occurs; at lower doses the weight loss can be minimized with some reduction in carcinostatic activity, but combined at these lower doses with 8-azaguanine it is reasonably effective. Therefore, it was

considered to represent a new class of potentially useful carcinostatic agents. It is also interesting that 6-aminonicotinic acid is one-seventh to one-fifteenth as toxic as the amide, suggesting either that penetration of the acid form is limiting or that conversion to the amide is slow.

In 1964 Herken et al showed that NADP is being converted into the nucleotide containing 6-AN faster and more completely than it is the case with NAD. Schultz et al (1966) reported that intraperitoneal injection of 6-AN (50 mg/kg) resulted in a marked hyperglycemia. They indicated that the hyperglycemic action of 6-AN was mediated by an increase of epinephrine release from the adrenal medulla. In brains of 6-AN treated rats, among the NADP-dependent oxidoreductases, the 6-phosphogluconate dehydrogenase was so strongly inhibited in vivo, that the tissue concentration of the 6-phosphogluconate rises to about the 200-fold of the normal level (Herken et al, 1969). The high intracellular 6-phosphogluconate caused an inhibition of the glycolysis on the level of the glucose-6-P isomerase for which 6-phosphogluconate is a competitive inhibitor in relation to glucose-6-P. Thus there is a direct inhibition of the hexose monophosphate pathway and an indirect inhibition of glycolysis. To gain further understanding of the relationship of the hexose monophosphate pathway to metabolic events in neural tissue, Kauffman and Johnson (1974) undertook additional studies of the effect of 6-AN on cerebral

energy metabolism. The accumulation of 6-phosphogluconate in brains of 6-AN-treated mice was dose-dependent; administration of 6-AN in doses of 10 and 35 mg/kg increased brain levels of 6-phosphogluconate 50-and 120-fold, respectively. Levels of ATP were reduced approximately 12% in the brain of 6-AN-treated mice with no change in levels of phosphocreatine. There was a five fold increase in cerebral glucose, and in spite of this the lactate levels were the same in the brains of 6-AN-treated and control mice. However, the decreased level of fructose-1, 6-diphosphate in cerebral tissue of 6-AN-treated mice indicates that metabolism via phosphofructokinase may have been inhibited in this tissue. Since brain levels of ATP decreased slightly after 6-AN treatment, the observed inhibition of phosphofructokinase would not appear to be due to this nucleotide. They calculated the total high-energy phosphate reserve in cerebral tissue of 6-AN-treated mice to be 35% higher than the control levels, which was accounted for by the increase in cerebral glucose and glycogen. They also measured the total amounts of NAD and NADP in neural tissues of 6-AN-treated and control mice. However, there were no significant differences in the pyridine nucleotide levels between normal and 6-AN-treated tissues. This is in agreement with Shapiro's report (1957) that 6-AN administration did not influence appreciably the NAD levels of liver and brain of treated animals. Recently Kehr et al (1978) investigated the effect of 6-AN on dopamine and noradrenaline synthesis and utilization in rat brain. In corpus

striatum, 6-AN (10 mg/kg i.p.) lowered the concentration of dopamine and markedly reduced the disappearance of dopamine after synthesis inhibition with α-methyl-p-tyrosine. They suggested that muscular rigidity produced by 6-AN may be associated with disruption of dopamine transmission in the striatum. Zeitz et al (1978) studied the effect of 6-AN on growth and acetylcholinesterase activity during differentiation of Neuroblastoma cells in vitro. They demonstrated that 6-AN causes a strong inhibition of cell division accompanied by higher cell volume, a decrease in protein content and an increase of specific acetylcholinesterase activity as compared to controls. It was suggested that 6-AN-induced differentiation may be accompanied by proteolytic processes.

Deficiency Symptoms

Pellagra has long been recognized as a disease resulting from nicotinic acid deficiency in humans. Deficiency signs include pigmentary changes in skin with loss of hair, loss of body weight, dementia, and alimentary manifestations such as anorexia and diarrhea. The pathological features are characteristic and consisted of chronic atrophic gastritis and degenerative changes in the mucosa of the small and large intestines. Mental symptoms form an important part of the clinical manifestations of pellagra. A study of the electroencephalographic (EFG) pattern showed that in most cases there was either

an excess theta activity or delta activity (Srikantia et al. (1968). The biochemical mechanisms underlying the mental change in pellagra have yet to be elucidated.

The main symptom of nicotinic acid deficiency in young chicks is an enlargement of the hock joint and bowing of the legs similar to perosis. The main difference between this condition and the perosis of manganese or choline deficiency is that in nicotinic acid deficiency the tendon of Achilles rarely slips from its condyles. Briggs (1946) described further symptoms of nicotinic acid deficiency as inflammation of the mouth, diarrhea, poor feathering and growth. On a niacin-low diet turkey poults develop an enlarged hock disorder. In ducklings its deficiency is characterized by lack of growth, diarrhea and general weakness. Growing rats, maintained on a low niacin diet, develop a condition characterized by growth depression.

2. PYRIDINE NUCLEOTIDES

Chemical Structures

1) NAD and NADH

empirical formula: NAD $C_{21}H_{27}O_{14}N_7P_2$

NADH $C_{21}H_{29}O_{14}N_7P_2$

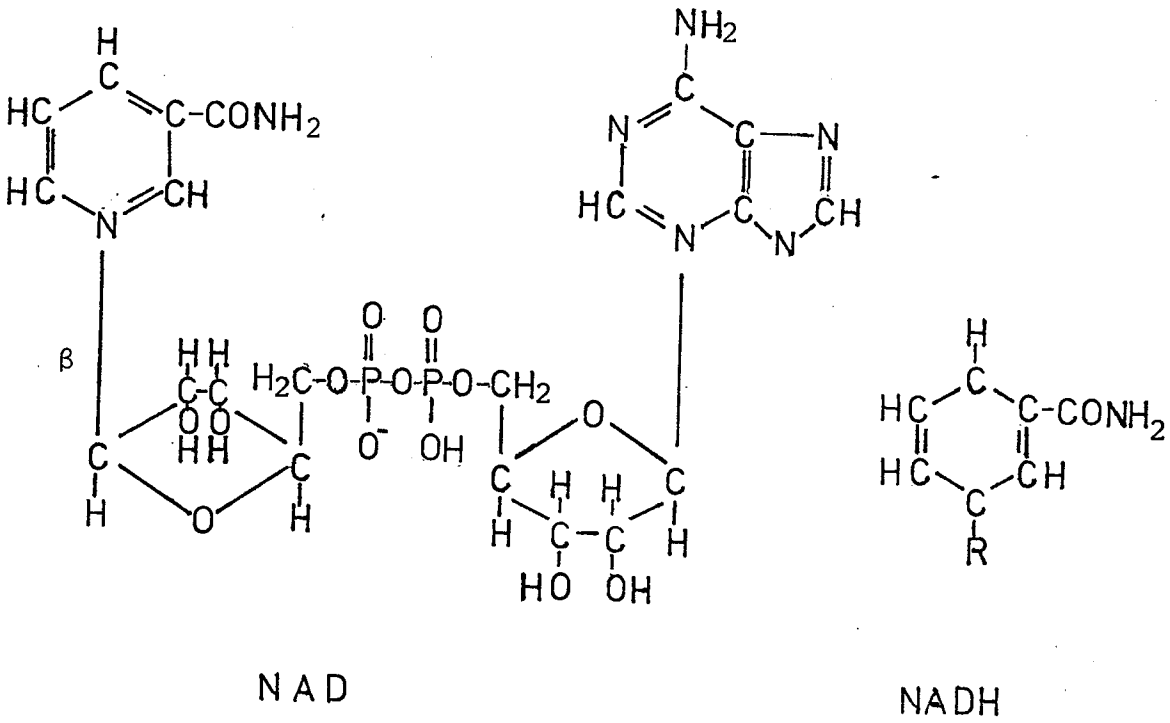


Figure 5. Chemical Structures of NAD and NADH.

molecular weight: NAD 663.3; NAD (ca.$4H_2O$) ca.735

NADH 665.3; NADH-Na_2(ca.$3H_2O$) ca.763

redox potential: E_O' =-0.318 V at pH 7.0 (Rodkey, 1955)

pK value: 3.9 (Kaplan et al, 1955)

Optical rotation $[\alpha]_D^{23}$:

ß-isomer; - 34.8°

α-isomer; + 14.3°

NAD is a white powder which is very soluble in water. It has only limited stability, but in the dry and cold state it keeps for months. Aqueous solutions at slightly acid pH are moderately stable, but unstable above pH 7.0. On the other hand, NADH is stable in dilute alkali (pH 7.5-12), but unstable in acids (Schlenk, 1951). Light and in particular heavy metals accelerate a partial oxidation of NADH solution to NAD. NADH is strongly hygroscopic compared to NAD. In moist preparations, inhibitors for dehydrogenases are formed without a decrease in $O.D._{340}$ and at the same time the preparations become colored, yellow to brown (McComb et al, 1968).

Like all nucleotides, NAD absorbs in UV light with a maximum at 260 nm; the corresponding molecular extinction coefficient is 18.0 x 10^6 cm^2/mole at pH 7.0. Aqueous solutions of NADH have slightly lower absorption at 260 nm than NAD solutions, but have a second characteristic absorption maximum at 340 nm. This is the basis of the enzymatic determination of NAD. A molecular extinction coefficient of 6.22 x $10^6 cm^2$/mole was obtained for NADH and NADPH at 340 nm (Horecker and Kornberg, 1948).

By measurements of the cyanide complex at 327 nm the sum of the pyridine coenzymes can be obtained; the difference between this value and that obtained enzymatically for β-NAD (measured at 340 or 365 nm) gives the content of α-NAD. α-NAD and β-NAD differ sterically in the glycosidic linkage between the nicotinamide and ribose. α-isomer can not be reduced enzymatically and is inactive as coenzyme. NAD fluoresces in strong alkali and this is the basis of the fluorimetric method for the determination of NAD after decomposition of NADH with acid (Kaplan et al, 1955; Lowry et al, 1957).

2) NADP AND NADPH

empirical formula: NADP $C_{21}H_{28}N_7O_{17}P_3$

NADPH $C_{21}H_{30}N_7O_{17}P_3$

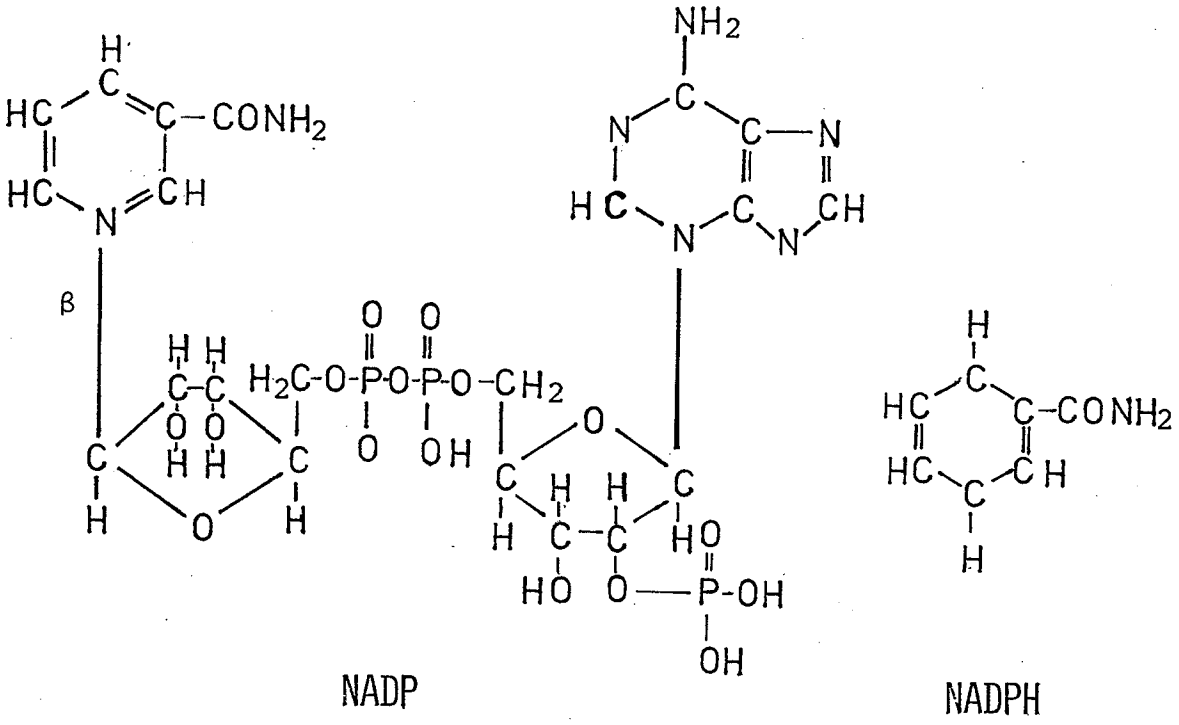


Figure 6. Chemical Structures of NADP and NADPH.

molecular weight: NADP 743.42 NADP-Na_2H 787.4

NADPH 745.42 NADPH-Na_4 834.0

Redox potential: E_o' =-0.317 V at pH 7.0 (Kaplan et al, 1953)

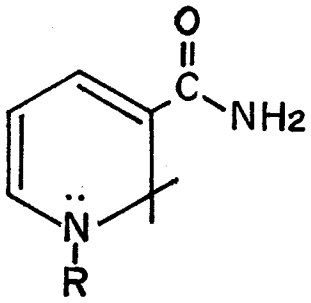
pK value: 6.2 - 6.3 (Theorell, 1934)

NADP and NADPH are colorless, hygroscopic compounds which are stable for several months when stored cool and dry, but which are sensitive to UV light. Although NADP is relatively stable in weak acids (pH 3-6), it is rapidly destroyed in alkaline solution with the cleavage of nicotinamide. NADPH is relatively stable in alkaline conditions (pH 8-9), but is destroyed on exposure to acid due to the opening of the pyridine ring (Schlenk, 1945). The addition compounds of NADP and acetone or other ketones exhibit a strong fluorescence in alkaline solution which can be used for the quantitative determination of NADP. As for NAD the α- and β-forms of NADP differ sterically in the glycosidic linkage between nicotinamide and ribose. The β-form only is enzymatically active (Kaplan et al., 1955).

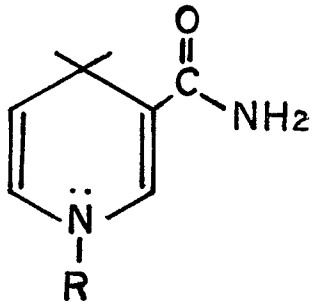
3) Stereospecificity for reduction of Pyridine coenzymes

As indicated above the conversion of NAD^+ or $NADP^+$ to the reduced form NADH or NADPH is accompanied by a marked alteration in the spectrophotometric properties of the coenzyme. The oxidized forms absorb maximally near 260 nm, the absorption being slightly less than the summation

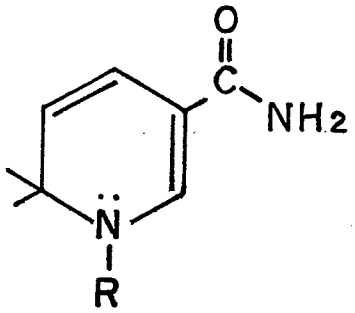
of that due to adenine and that due to nicotinamide. On reduction, the absorbance at 260 nm decrease considerably, and a new band having a maximum at 340 nm, characteristic of a dihydronicotinamide, appears. Thus the nicotinamide moiety of these coenzymes is the site of the oxidation-reduction reactions. Electronically, reduction might take place at either the 2, 4, or 6 position of the nicotinamide ring, yielding the corresponding dihydro derivatives (I, II, and III): (Pullman and Colowick, 1954a).



(I)



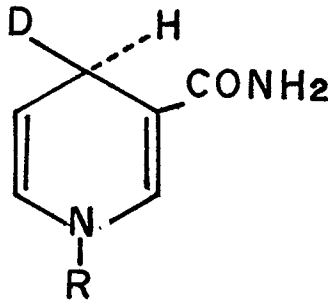
(II)



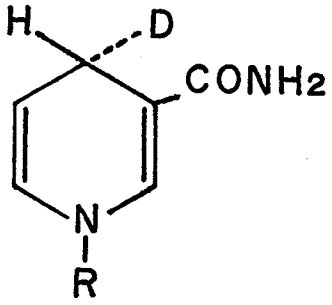
(III)

Taking advantage of the fact that the ferricyanide oxidation of N'-methylnicotinamide is known to yield predominantly the 2- and 6-pyridones, Pullman et al (1954b) demonstrated that position 4 is the actual site of reduction. The conversion of NAD^+ to NADH results in the formation of a new asymmetric center. Dithionite reduction of NAD^+ in

D_2O medium yields the two stereoisomers as follows:



(A)



(B)

Forms A and B differ only in the position occupied by the deuterium with respect to the plane of the pyridine ring. NAD^+- and NADP-linked dehydrogenases exhibit stereospecificity for the A or B form of the reduced coenzyme. This was convincingly demonstrated by Vennesland et al (1954) for alcohol dehydrogenase(ADH) and LDH. Deuterium-labelled reduced NAD^+ was prepared enzymatically with ADH and 1.1-dideuterio-ethanol: $CH_3CD_2OH + NAD^+ \longrightarrow CH_3CDO + NADD$ When the reduced coenzyme was reoxidized with either acetaldehyde and ADH or pyruvic acid and LDH, the deuterium was quantitatively removed from the coenzyme and transferred to the reduced substrate. These experiments present the following points. First, the transfer of hydrogen between substrate and coenzyme is direct. Second, ADH and LDH are sterospecific with respect to coenzyme. Finally, both ADH and LDH transfer hydrogen to and from the same side of the NAD^+ molecule (form A). Some enzymes utilize form A, some form B. In only one known case, that of diaphorase (lipoyl dehydro-

genase), is hydrogen exchanged on both sides of the nicotinamide ring, and this enzyme contains a flavin as prosthetic group. Formally, NAD^+- and NADP-dependent reactions involve the transfer of a hydride ion, a hydrogen nucleus with two electrons, between substrate and coenzyme. Such reactions may proceed via an actual transfer of a hydride ion or via a two-step reaction pathway involving the transfer of a hydrogen atom preceded or succeeded by the transfer of an electron. Although it is not known with certainty which of these alternatives, singly or in combination, correctly describe NAD^+-dependent enzymatic reactions, the hydride ion transfer mechanism appears more likely. The overall kinetics and magnitude of the kinetic deuterium isotope effect in some of the enzyme-catalyzed cases are consistent with the hydride ion mechanism (Abeles et al, 1957; Abeles and Westheimer, 1958).

Biochemical Functions of NAD

1) Carbohydrate Metabolism

(a) Anaerobic and Aerobic Oxidation of Glucose

(i) glyceraldehyde-3-phosphate dehydrogenase (Krebs, 1955)

D-glyceraldehyde-3-P + NAD^+ + P_i ⟶ 1,3-diphosphoglycerate + NADH + H^+

This reaction is the first one in the glycolytic sequence to involve oxidation reduction which results in conservation of the energy of oxidation of the aldehyde group of glyceraldehyde-3-P in the form of a high energy phosphate compound formed as 1,3-diphosphoglycerate.

(ii) Lactate dehydrogenase (Fritz, 1965)

Pyruvate + NADH + H^+ ⟷ lactate + NAD^+

In the last step of glycolysis, pyruvate is reduced to lactate at the expense of electrons originally donated by glyceraldehyde-3-P under anaerobic conditions

(b) TCA cycle

(i) pyruvate dehydrogenase (Koike et al, 1963)

pyruvate + NAD^+ + CoA-SH ⟶ acetyl-S-CoA + NADH + H^+ + CO_2

Although it is not itself part of the TCA cycle, it is an obligatory step by which carbohydrates (via pyruvate) enter the cycle.

(ii) isocitrate dehydrogenase (Chen and Plaut, 1962)

$$\text{Isocitrate} + NAD^+ \ (NADP^+) \rightleftharpoons \alpha\text{-ketoglutarate} + CO_2 + NADH \ (NADPH) + H^+$$

The oxidation of isocitrate to α-ketoglutarate is catalyzed by an allosteric enzyme and is usually the rate-limiting reaction of the TCA cycle.

(iii) α-ketoglutarate dehydrogenase (Massey, 1960)

$$\alpha\text{-ketoglutarate} + NAD^+ + \text{CoA-SH} \rightleftharpoons \text{succinyl-S-CoA} + CO_2 + NADH + H^+$$

The reaction as a whole is not readily reversible because of the decarboxylation step.

(iv) malate dehydrogenase (Callahan and Kosicki, 1967)

$$\text{L-malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH + H^+$$

In the last reaction of the cycle, the NAD-linked L-malate dehydrogenase catalyzes the oxidation of L-malate to oxoloacetate.

(c) HMP Shunt Pathway

(i) glucose-6-P dehydrogenase (DeMoss, 1955)

$$\text{glucose-6-P} + NADP^+ \rightleftharpoons \text{6-phosphogluconate} + NADPH + H^+$$

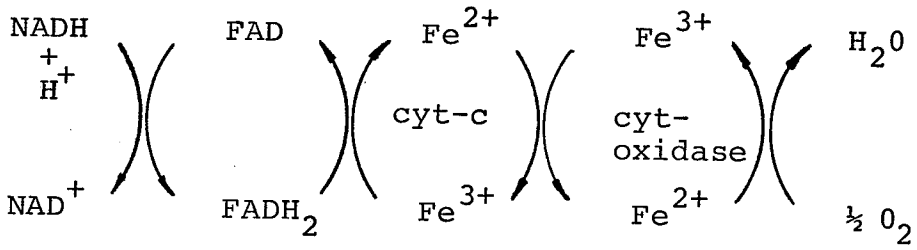
(ii) 6-phosphogluconate dehydrogenase (Pontremoli and Grazi, 1966)

$$\text{6-phosphogluconate} + NADP^+ \longrightarrow \text{D-ribulose-5-}\underline{P} + NADPH + H^+ + CO_2$$

HMP shunt pathway provides NADPH required by anabolic processes outside the mitochondria.

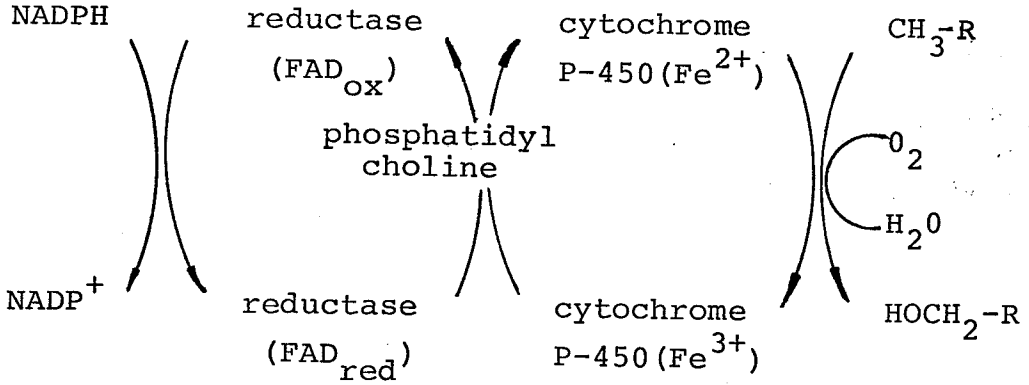
2) Electron Transport System

(i) Oxidative Phosphorylation (Lehninger, 1975)



3 moles of ATP are formed per mole of NADH oxidized or atom of oxygen consumed.

(ii) Microsomal hydroxylation reaction (Dus, 1976)



Microsomal hydroxylases contain electron transport systems capable of utilizing molecular oxygen to oxidize specific organic molecules, by direct insertion of one or both its oxygen atoms into the product.

3) Lipid Metabolism

(a) Triglyceride synthesis

glycerol-phosphate dehydrogenase (Beisenherz et al, 1955)

$$\text{Dihydroxyacetone phosphate} + \text{NADH} + H^+ \rightleftharpoons \text{L-glycerol-3-P} + NAD^+$$

(b) Fatty acid oxidation and synthesis

(i) β-ketoacyl-ACP-reductase (Majerus, 1965)

$$\text{acetoacetyl-S-ACP} + \text{NADPH} + H^+ \rightleftharpoons \text{D-β-hydroxybutyryl-S-ACP} + NADP^+$$

(ii) crotonyl-ACP-reductase

$$\text{crotonyl-S-ACP} + \text{NADPH} + H^+ \rightleftharpoons \text{butyryl-S-ACP} + NADP^+$$

(iii) L-3-hydroxyacyl CoA dehydrogenase

$$\text{L-3-hydroxyacyl CoA} + NAD^+ \rightleftharpoons \text{3-ketoacyl CoA} + \text{NADH} + H^+$$

(c) Steroid synthesis

(i) β-hydroxy-β-methylglutaryl CoA reductase

$$\text{β-hydroxy-β-methylglutaryl CoA} + 2\ \text{NADPH} + 2H^+ \longrightarrow \text{mevalonic acid} + 2\ NADP^+ + \text{CoA}$$

(ii) Squalene synthetase

$$2\ \text{farnesyl pyrophosphate} + \text{NADPH} + H^+ \longrightarrow \text{squalene} + NADP^+ + 2PP_i$$

(d) Interconversion of acetoacetate and β-hydroxybutyrate

(i) β-hydroxybutyrate dehydrogenase

$$\text{acetoacetate} + \text{NADH} + H^+ \rightleftharpoons \text{β-hydroxybutyrate} + NAD^+$$

(e) Malic enzyme (Hsu and Lardy, 1969)

$$\text{L-malate} + NADP^+ \rightleftharpoons \text{pyruvate} + CO_2 + NADPH + H^+$$

NADPH generated is another source of reducing power for fatty acid synthesis in liver.

4) Protein Metabolism

(a) Degradation and synthesis of amino acids

(i) phenylalanine hydroxylase (Kaufman, 1957)

$$\text{phenylalanine} + NADPH + H^+ + O_2 \longrightarrow \text{tyrosine} + NADP^+ + H_2O$$

(ii) glutamate dehydrogenase (Sedgewick and Frieden, 1968)

$$NH_3 + \alpha\text{-ketoglutarate} + NADPH + H^+ \rightleftharpoons \text{L-glutamic acid} + NADP^+$$

(b) glutathione reductase (Colman and Black, 1965)

$$2GSH + NADP \rightleftharpoons GSSG + NADPH + H^+$$

5) Vitamin Metabolism

(a) Ascorbic acid synthesis

(i) glucuronate reductase (Chaudhuri and Chatterjee, 1969)

$$\text{D-glucuronic acid} + H_2O + NADPH + H^+ \rightleftharpoons \text{L-gulonic acid} + NADP^+$$

This gulonic acid is the direct precursor of ascorbic acid in those animals which are capable of synthesizing this vitamin.

(b) Vitamin A synthesis

(i) Retinene reductase

$$\text{retinal} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{retinol} + \text{NAD}^+$$

(c) Tetrahydrofolic acid synthesis

(i) folic reductase

$$\text{folic acid} + \text{NADPH} + \text{H}^+ \longrightarrow \text{FH}_2 + \text{NADP}^+$$

(ii) dihydrofolic reductase (Stanley et al, 1971)

$$\text{FH}_2 + \text{NADPH} + \text{H}^+ \longrightarrow \text{FH}_4 + \text{NADP}^+$$

Before functioning as a C_1 carrier or a formate unit, folic acid must be reduced using NADPH by both enzymes above.

6) Other functions (as a substrate for ADP-ribosylatron)

The major biochemical function of NAD is known to be as an coenzyme in various biological oxidation-reduction systems. The structure of NAD can also be envisaged as the adenosine diphosphate-ribosyl moiety (ADP-ribose) attached covalently to a nicotinamide through a β-N-glycosidic linkage. This linkage constitutes a so-called high-energy bond, since its free energy of hydrolysis is approximately -8.2 kcal/mole at pH 7.0 and 25°C. The energy of this bond supplies the driving force for the various ADP-ribosylation reaction.

Two kinds of NADase have been reported. Microbial NADase hydrolyze the N-glycosidic linkage of NAD, yielding free ADP-ribose, nicotinamide, and a proton. Mammalian

NADase, in contrast, catalyze both hydrolysis of NAD and transfer of the ADP-ribose moiety to various basic acceptors, thus acting as both hydrolases and trans-glycosidases. One such reaction is the diphtheria toxin (fragment A)-catalyzed transfer of ADP-ribose from NAD to eukaryotic EF2 (elongation factor), resulting in the covalent modification and selective inactivation of this factor and the concomittant inhibition of protein synthesis (Pappenheimer, 1977). Another ADP-ribose transfer reaction involves the functioning of NAD as a substrate for poly (ADP-ribose) synthetases. Studies of this reaction have suggested a close relationship between poly(ADP) ribosylation and the control of gene expression. Poly (ADP-ribose) synthetase is tightly associated with chromatin and catalyzes the synthesis of homopolymer poly (ADP-ribose) from the ADP-ribosyl moiety of NAD.

Burzio and Koide (1970) reported that the treatment of isolated rat liver nuclei or chromatin with NAD decreased the incorporation of ^{3}H-labelled deoxyribonucleotides into DNA due to the formation of poly (ADP-ribose). In contrast to the findings of Burzio and Koide, Roberts et al (1973) demonstrated the activation of DNA synthesis in Hela cells. Thus, it was concluded that the effect of poly (ADP)-ribosylation on nuclear DNA synthesis is apparently dependent on the type of cells involved. Evidence suggesting a crucial role for poly ADP-ribosylation in both S and G_2 stages has been presented by Kidwell et al (1974). They

showed from pulse-labelling mouse L cells with [^{3}H]-adenosine that there are two short-lived bursts of poly (ADP-ribose) synthesis during the S and S to G_2 phases, while the rate of DNA synthesis increases continuously. A nicotinamide, an inhibitor of poly (ADP-ribose) synthetase, diminished poly (ADP-ribose) synthesis *in vivo* and also DNA synthesis, but the latter effect was apparent only after the time of the first burst of poly (ADP-ribose) synthesis. These observations suggest that DNA replication may require an immediately prior synthesis of poly (ADP-ribose). These results, together with other reports, indicate that poly (ADP)-ribosylation functions in the G_2 phase in some way to sustain continuous cell proliferation. Recently Lorimer *et al* (1976) reported that poly (ADP-ribose) appears to cross-link two H1 histone molecules to make a dimer-polymer complex. Based on these observations, they postulate that poly (ADP-ribose) functions as a bridge in the reversible condensation of chromatin via a linkage of neighboring H1 molecules. Furthermore Smulson *et al* (1976) demonstrated that histones derived from nuclei preincubated with NAD have less affinity for DNA than unmodified histones. These data, taken together, appear to support the hypothesis that the covalent attachment of poly (ADP-ribose) to histones alters their structure and results in their decreased affinity for DNA.

Little informations are available regarding the correlation between the NAD concentration and the activity of poly (ADP-ribose) synthetase in various tissues with different proliferation rates. Recently it was revealed that the nucleus is the major locus of NAD breakdown in Hela cells and that the cleavage occurs exclusively at the nicotinamide-ribose linkage (Rechsteiner, 1976). These findings are in accord with the earlier observations indicating rapid turnover of NAD as well as poly (ADP-ribose) in nuclei, and suggest a crucial role for poly (ADP)-ribosylation in cellular NAD metabolism (Roberts, 1974). Caplan et al (1976) recently presented evidence that the cellular NAD level may be a determinant for differentiation of embryonic chick mesodermal cells into muscle or cartilage and that poly (ADP-ribose) synthesis is closely related to this differential phenotypic expression; they suggested that poly (ADP)-ribosylation of histones is involved in the control mechanism.

Distribution of pyridine nucleotides (or coenzymes)

The nicotinamide dinucleotides, NAD, NADH and nicotinamide dinucleotide phosphates NADP, NADPH occur in all living cells. Within cells, the nicotinamide dinucleotides are not distributed evenly over the various subcellular regions, e.g. mitochondria and cytosol. In whole blood, the nicotinamide dinucleotides occur only within the cell elements

(erythrocytes, thrombocytes, leucocytes). Glock and McLean (1955) reported that NADPH is present in great excess over NADP in whole rat liver, whereas NAD is present in higher concentrations than NADH in the same tissue. The only other tissue besides liver which shows significant concentrations of the NADPH is the adrenal cortex. Jedeikin and Weinhouse (1955) demonstrated that skeletal muscle contains roughly the same amount of NAD as liver does. However, the level of NADH in skeletal muscle was found to be relatively low. McLean (1958) also reported an interesting finding with regard to the concentration of pyridine coenzymes in the rat mammary gland. She observed a remarkable rise in the level of NAD and NADPH during lactation and a sharp fall during the involution of the gland. Jacobson and Kaplan (1957) indicated that approximately 10% of the total pyridine coenzymes of rat liver can be found in the mitochondria. Mitochondria fractions contain predominently NADPH and very little NADP; on the other hand, NAD is usually found in higher levels than is NADH. It was also reported that the concentration of pyridine coenzymes in microsomes is considerably less than in mitochondria, and the coenzymes are primarily in the oxidized form. In addition, nuclei contains very little of either reduced or oxidized forms of the coenzymes. The rat heart contains more total pyridine coenzymes than either brain or kidney but less than the liver; the heart is also similar to brain and kidney in having a smaller amount of reduced coenzymes than oxidized ones in the mitochondria. Jacobson and Kaplan

(1957) also compared the level of mitochondrial pyridine coenzymes among species. Pigeon and mouse liver homogenate contain amounts of oxidized and reduced pyridine coenzymes comparable to those of rat liver homogenate. On the other hand, examination of the mitochondria from both the mouse and pigeon liver showed that the pyridine coenzyme distribution was quite different from that of rat liver. The divergence was greater in the case of pigeon liver mitochondria in which the oxidized pyridine coenzymes exceeded the reduced forms by 15 times; in the case of mouse liver mitochondria, the oxidized exceeded reduced coenzymes by 1.5 to 2.0 times. In the mitochondria of rat liver the ratio is reversed (reduced to oxidized) to give values of 2 to 3.

NAD Biosynthesis and degradation

The biosynthesis of pyridine nucleotides and the regulation of their intracellular concentrations are complicated by the existence of three different precursors such as tryptophan, nicotinic acid, and nicotinamide. Several studies have clearly implicated tryptophan and its metabolites as precursors of niacin in mammals and *Neurospora* (Partridge *et al.*, 1952). In addition, Nishizuka *et al* (1963) have shown that quinolinic acid is a key intermediate in the conversion of tryptophan to nicotinic acid mononucleotide in rat liver (reaction 4, Fig. 7.). As can be seen in Figure 7., quinolinic acid is synthesized from tryptophan or *de novo* from amino acid and carbohydrate intermediates. The synthesis of quinolinic acid from

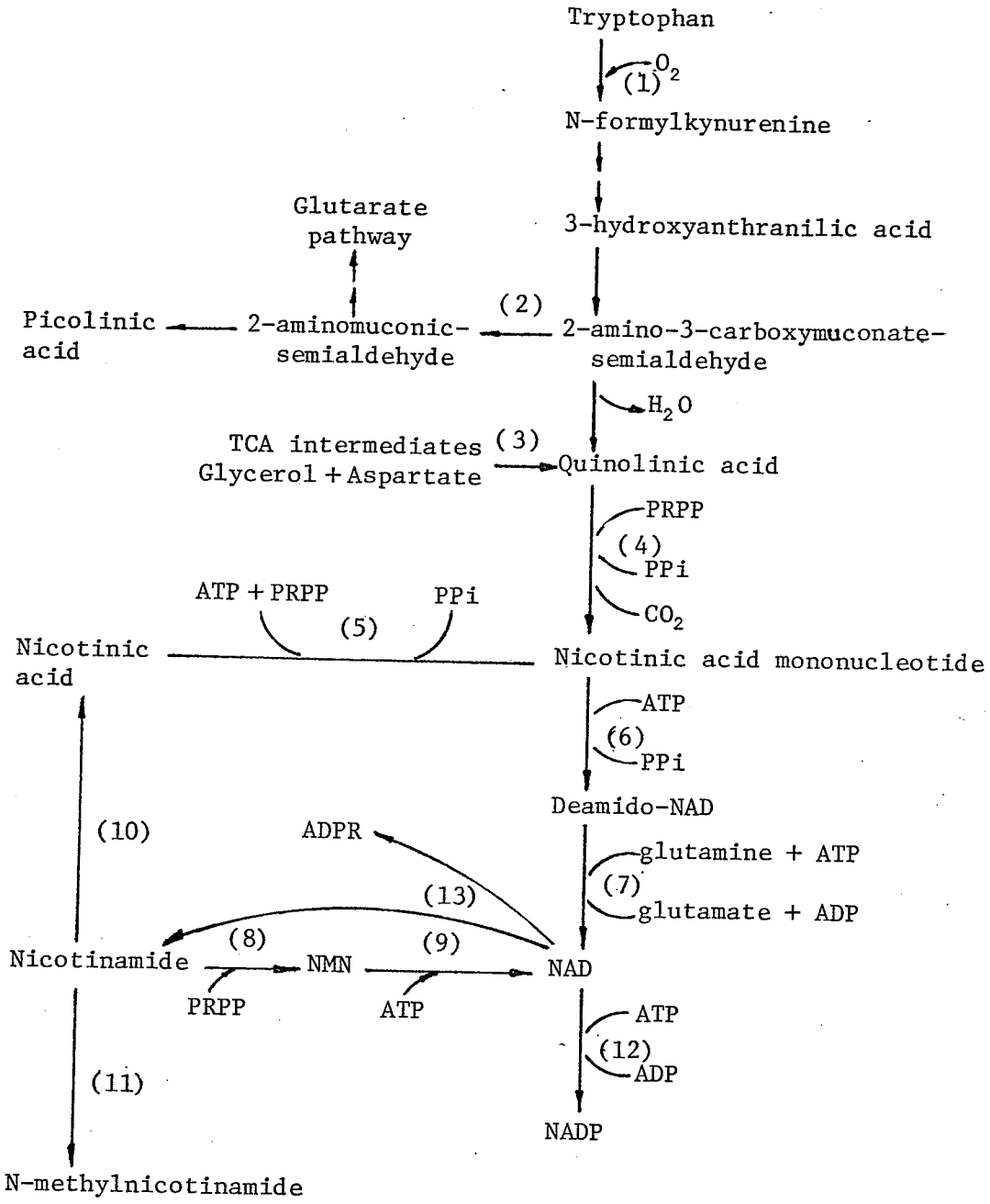


Figure 7. Pathways of synthesis and degradation of NAD.

Figure 7. Continued.

Enzymes involved in reaction (1)-----(13) :

(1) Tryptophan pyrrolase

(2) Picolinic carboxylase

(4) Quinolinate phosphoribosyltransferase

(5) Nicotinate mononucleotide phosphoribosyltransferase

(6) Nicotinate phosphoribosyltransferase

(7) NAD synthetase

(8) Nicotinamide mononucleotide phosphoribosyltransferase

(9) NAD pyrophosphorylase

(10) Nicotinamide deamidase

(11) Nicotinamide methyltransferase

(12) NAD kinase

(13) NADase (NAD glycohydrolase)

compounds other than tryptophan (reaction 3, Fig. 7) is apparently restricted to microorganisms and plants.

The tryptophan-NAD pathway is very important because it may be the ultimate source of NAD in mammalian cells in the absence of nicotinic acid and nicotinamide. Control of NAD synthesis from tryptophan appears to be exerted at the tryptophan pyrrolase and picolinic carboxylase steps. Tryptophan pyrrolase is the first enzyme (an irreversible one) on the tryptophan-NAD pathway, and its activity determines how much tryptophan enters the pathway (reaction 1, Fig. 7). Cho-Chung et al (1967) reported that the activity of the purified tryptophan pyrrolase is allosterically inhibited by NADPH and also by NADH. Since these 2 reduced pyridine nucleotides are the end products of tryptophan metabolism via the kynurenine pathway, they suggested that the tryptophan pyrrolase may be regulated by a feedback mechanism

Ikeda et al (1965) have reported that picolinic carboxylase can be another factor which determines whether 2-amino-3-carboxymuconate semialdehyde, derived from 3-hydroxyanthranilic acid, proceeds into the quinolinic pathway leading to NAD^+ synthesis, or into the glutarate pathway (reaction 2, Fig.7). The latter results in the complete oxidation of the tryptophan molecule. Picolinic carboxylase activity seems to be genetically regulated. Its activity in cat liver is about 30 to 50 times higher than in the liver of rat and man, so that in the former

species 2-amino-3-carboxymuconate semialdehyde is decarboxylated before it can enter the NAD pathway (Ichiyama et al, 1967). This is in agreement with the fact that tryptophan can not replace nicotinamide in the diet of cats. Furthermore, there seems to be a hormonal control of picolinic carboxylase activity, which has been found to be increased in the livers of alloxanized or pancreatectomized rats. Insulin administration returns the activity of this enzyme to the normal level (Mehler et al, (1965).

The other pathways of NAD biosynthesis, which employ nicotinic acid or nicotinamide as precursors, have been investigated by Preiss and Handler (1958). They described the biosynthetic pathway of nicotinic acid to NAD including nicotinic acid mononucleotide and deamido-NAD as intermediates of this metabolic chain (reactions (5), (6), and (7), Fig. 7). A prominent feature of this sequence is the fact that all 3 steps result in the formation of inorganic pyrophosphate. For the formation of NAD from nicotinamide 3 possibilities exist: (a) the pathway via nicotinamide and NMN (reactions (8) and (9), Fig. 7), (b) the pathway via nicotinamide, nicotinic acid, nicotinic acid mononucleotide and deamido-NAD (reactions (10), (5), (6) and (7), Fig. 7) and (c) the transfer reaction catalyzed by the NADase (reaction (13), Fig. 7).

In addition, Preiss and Handler (1957) also have reported that the synthesis of NMN from nicotinamide and PRPP in human erythrocytes. However, they found that NMN phos-

phoribosyltransferase which can initiate the net synthesis of NAD from nicotinamide exhibit a high K_m for nicotinamide in the order of 0.1 M. The range of nicotinamide concentrations considered "physiological" is 5×10^{-5} to 10^{-6}M. This observation led them to conclude that the formation of NAD from nicotinamide may not take place via NMN under physiological conditions. In contrast to Preiss's findings, Dietrich et al (1966) reported that NMN phosphoribosyltransferase is present in livers of rats with an apparent K_m of 2.6×10^{-6}M for nicotinamide. Thus the discovery by Dietrich et al (1966) of a NMN phosphoribosyltransferase with a high affinity for nicotinamide has raised the possibility of the direct utilization of nicotinamide in the biosynthesis of NAD. In support of Dietrich's findings, Ohtsu et al. (1967) have found that Lactobacillus fructosue appears to form NAD from nicotinamide via NMN without being converted to nicotinic acid. Also Grunicke et al (1966) observed that NAD biosynthesis from nicotinamide via NMN represents an appreciable part of the total NAD formation in Ehrlich ascites tumor cells. A similar observation was made by Greenbaum and Pinder (1968) for mammary tissue. Dietrich et al. (1968) have demonstrated that NMN phosphoribosyltransferase is subject to feedback control by NAD, NADP, and NADPH at their normal concentrations. This enzyme is also controlled by the ATP concentration, ATP probably serving as a positive modifier of the enzymic reaction. On the other hand, Streffer and Benes (1971) reported that the biosynthesis of NAD was mainly via the

nicotinic acid pathway and only a small fraction of nicotinamide is incorporated directly to NAD via NMN after the intraperitoneal application of a high nicotinamide dose (1 mmole/kg body weight). However, after the injection of a small nicotinamide dose within the physiological range the pathway via NMN is utilized to a large extent. Their data demonstrated that NAD biosynthesis from nicotinamide is almost independent of the nicotinic acid pathway when the level of the nicotinamide in the liver tissue is low or comparable to the physiological situation. According to Wang and Kaplan's reports (1954), NADP was synthesized from NAD by the NAD kinase reaction in which NADH did not serve as a substrate (reaction 12, Fig. 7).

Hughes and Williamson (1953) demonstrated the existence of nicotinamide deamidase in washed suspensions and cell-free extracts of Lactobacillus arabinosus which results in the hydrolysis of nicotinamide to nicotinic acid and ammonia. According to Chaykin et al (1965) nicotinamide is the predominant form of the vitamin in blood and other mammalian tissues. Kaplan and his co-workers (1956) indicated that when nicotinamide was administered to mice intraperitoneally the NAD level in the liver increased markedly, presumably because of inhibition of NADase by nicotinamide. Moreover, nicotinic acid has been shown to give a much smaller rise in NAD than does nicotinamide. Petrack et al (1963) have isolated nicotinamide deamidase from liver in rat and also suggested that the greater efficacy of nicotinamide than

of nicotinic acid for the synthesis of liver NAD *in vivo* might be due to the long half-life of nicotinamide (4½ hours) compared to nicotinic acid (1 hour). The long half-life of nicotinamide, together with the occurrence of nicotinamide deamidase in mammalian liver, permits the generation, over a prolonged period of time following nicotinamide injection, of a noninhibitory level of nicotinic acid, which is used for the synthesis of NAD.

Considering the reported high K_m value (4×10^{-2} to 2.5×10^{-1}M) of nicotinamide deamidase for nicotinamide and the presence of an inhibitor in liver tissue which masks the activity of nicotinamide deamidase Hagino *et al* (1968) suggest that this enzyme may play little or no role in the intact liver. However, Hayaishi and co-workers (1966) presented some evidence that the Preiss-Handler pathway is functional in animals lacking deamidase in the liver because nicotinamide is not deamidated in the liver. They approached the problem with the pulse labelling technique and demonstrated the involvement of both the liver and the intestine in the conversion of nicotinamide to NAD. They observed that nicotinamide administered by intraportal or intraperitoneal injection first passes into the intestine and is deamidated by the action of the bacterial flora or gastrointestinal enzymes. The resulting nicotinic acid was then absorbed and transported to the liver where it was converted to NAD by the reactions described by Preiss and Handler (1958). They also observed that nicotinic acid-^{14}C

is a better precursor of NAD than nicotinamide when administered in small doses (78 mμmoles). When a large dose of nicotinamide-^{14}C (82 to 164 μmoles) was injected, however,the liver NAD level increased markedly for a prolonged period of time. Such an observation confirms the previous reports by Kaplan and his co-workers (1956). In contrast, injection of nicotinic acid-^{14}C in a large dose gave a much smaller rise in liver NAD than did injection of nicotinamide-^{14}C. Greengard et al (1964) showed that in hypophysectomized rats injection of nicotinamide leads to a much larger and more prolonged rise in hepatic NAD levels than in normal rats. They proposed that this effect may be due, at least in part, to an increased hepatic nicotinamide deamidase activity brought about by the absence of an endogenous inhibitor of this enzyme in hypophysectomized rats. The inhibitor appears to act by increasing the K_m for nicotinamide.

An important feature of the metabolic pathway of NAD is the involvement of NADase, the enzyme that cleaves NAD and NADP at the nicotinamide riboside linkage. The NADase reaction is irreversible and NAD can not be synthesized from adenosine diphosphate ribose and nicotinamide; this is due to the fact that the nicotinamide-riboside linkage is a high energy bond. This enzyme is almost ubiquitously distributed in animal tissues. It is found in the highest concentrations in lung and spleen (Quastel et al, 1953). However, muscle contains the lowest concentration of the

enzyme. Also the NADase activity is generally concentrated in the microsomal fraction of liver, kidney, and brain.

Kaplan (1968) found that the NADase has a low activity on NAD which is bound to dehydrogenases; this inactivity on the bound coenzyme may be an important factor in the regulation of the level of the coenzyme. In conclusion, Kaplan (1968) indicated that the regulation of pyridine coenzyme levels in animal tissues is controlled by (a) the level of synthetic enzymes for pyridine coenzymes, (b) the NADase, (c) the amount of pyridine nucleotide-linked enzymes. Gholson (1966) emphasized the importance of efficient re-utilization of nicotinamide released from NAD by the action of NADase. On the basis of this observation he proposed the so-called "pyridine nucleotide cycle" would be of use to all organisms in maintaining the adequate coenzyme concentrations in the cell. The schematic outline of the systemic pyridine nucleotide cycle is shown in Fig. 8. Tryptophan and nicotinic acid released from dietary proteins in the gastrointestinal tract are synthesized into NAD in liver, which is then degraded via NADase, releasing nicotinamide that is then excreted back into the general circulation. Nicotinamide, on the other hand, might also be excreted back into the gastrointestinal tract, be converted there into nicotinic acid by the intestinal flora of the tract. The cycle is completed by the reassimilation of the released nicotinic acid into NAD by appropriate enzymes.

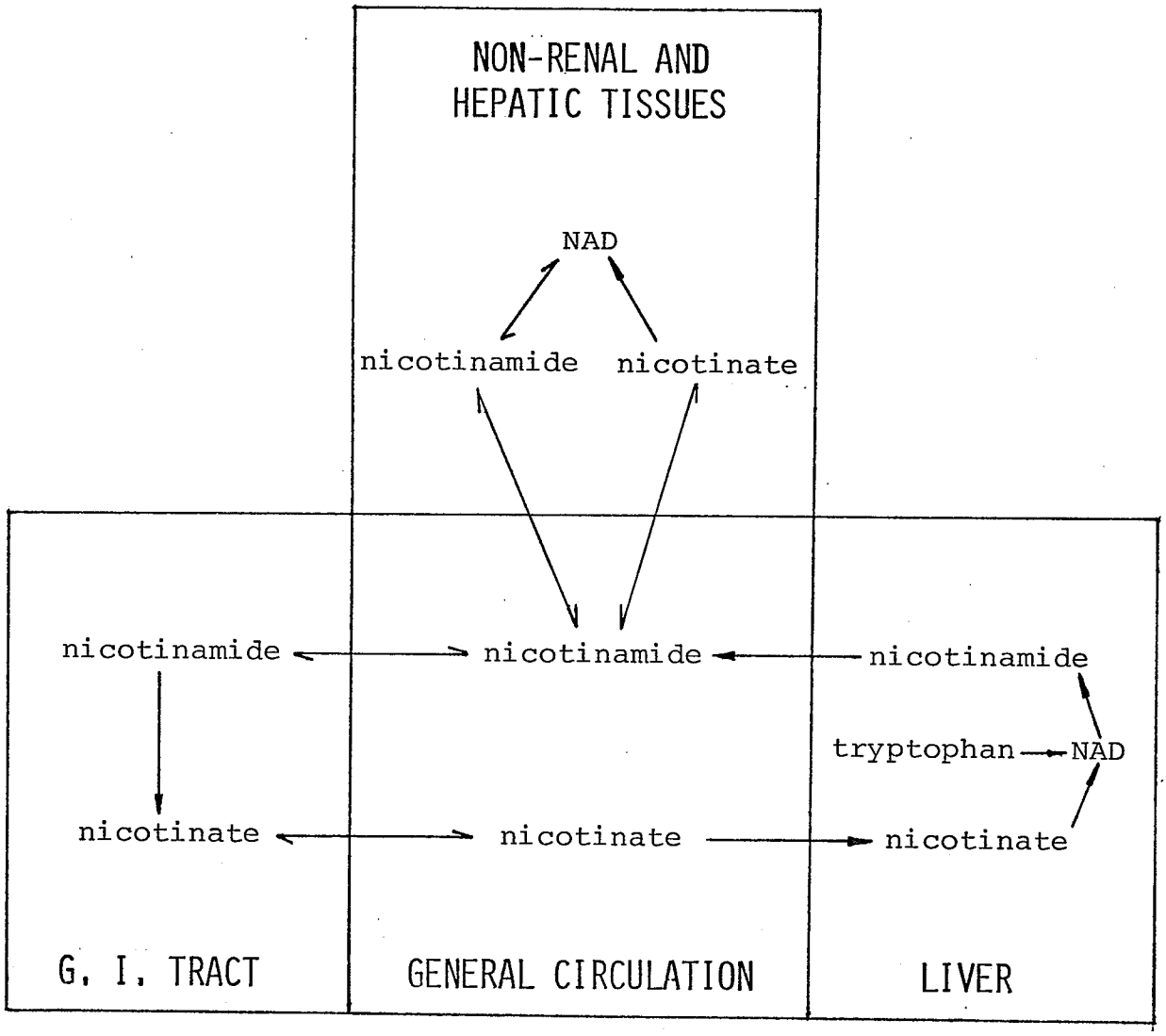


Figure 8. Systemic pyridine nucleotide cycle.

Gerber and Deroo (1970) subsequently investigated the incorporation of labelled nicotinic acid and nicotinamide into NAD and replacement of labelled NAD in different organs of mice and rats. Following intraperitoneal injection of ^{14}C-nicotinic acid or ^{14}C-nicotinamide in rats and mice, they observed that at all times later than 3 hr after injection, NAD represented 70-80% of the radioactivity in all the tissues studied and that the only other important radioactive metabolite present was nicotinamide. Surprisingly, nicotinamide mononucleotide, nicotinic acid mononucleotide, NADP and deamido-NAD contained no significant radioactivity. They also showed that the replacement of NAD was rapid in kidney, liver, and intestine, and slow in brain, muscle, and testis. The value found for replacement of liver NAD was in good agreement with the half-life of 10 hr reported by Ijichi et al (1966) for mouse liver NAD. Since free radioactive nicotinic acid disappears rapidly from all tissues, it has been suggested that extensive reutilization of breakdown products of NAD, i.e., nicotinamide, takes place, and that this effect may differ from one organ to another. Deguchi et al (1968) explored the biosynthesis of NAD in the brain by the administration of various radioactive precursors directly into the cisternal cavity of rats. They observed that the brain is capable of synthesizing NAD rapidly and efficiently from nicotinic acid by way of nicotinic acid mononucleotide and deamido-NAD, as described in the liver and erythrocytes.

The rate of synthesis in the brain was comparable to that in the liver. However the half-life of NAD in brain was 3-4 days compared to approximately 10 hr in liver. This paradox between the relatively high synthetic activity and low turnover rate of brain NAD seems to be ascribed to the limited penetration of NAD precursors across the blood-brain barrier.

Very recently Rechsteiner et al (1976) presented data for D98/AH2, a human cell line derived from Hela which makes possible calculation of the fraction of NAD biosynthesis that compensates for breakdown. D98/AH2 cells were grown in medium containing ^{14}C-adenine and ^{3}H-nicotinic acid, both precursors of NAD, for 6 hr and then moved to unlabelled medium. At various times after transfer, they extracted intracellular nucleotides and calculated the breakdown rate of NAD in vivo. They demonstrated that the half-life of an intact NAD molecule is approximately 1 hr in whole D98/AH2 cells, yet in enucleated cells the half-life of an intact NAD molecule is 10 hrs. They suggested that NADase and poly ADPR synthetase may be involved in NAD turnover in mammalian cells. However, the relative contributions of these two enzymes to NAD turnover are still unknown. Nevertheless, it has been speculated that the formation of the unusual nuclear polymer, poly-ADPR, is probably the major source of NAD turnover in mammalian cells. Furthermore, Rottman et al (1974) postulated the possibility that NAD turnover is involved in some aspects of RNA processing such as the modifications at the 5'end of mRNA molecules.

Effect of Niacin on the level of pyridine nucleotides

Kohn (1938) has demonstrated an increase in the blood factor V (coenzyme I or coenzyme II) of both normal subjects and pellagra patients following the ingestion of large amounts of nicotinic acid and Vilter et al (1939) have observed a decrease in the factor V content of the blood of pellagrins which could be remedied through nicotinic acid therapy. Axelrod et al (1939), using a yeast fermentation method, made the following observations: (1) a nicotinic acid deficiency in dog and pig results in a lowered coenzyme I content of the liver and muscle (2) no effect is noted upon the coenzyme I content of the brain, kidney cortex and blood. (3) The coenzyme I content of rat tissues can not be increased by the administration of excessive amounts of nicotinic acid. Von Euler et al (1938) also made a similar observation that there is no increase in the coenzyme I content of the tissues of rats fed excessive nicotinic acid whereas Dann and Kohn (1940) reported only a slight increase. Axelrod et al (1941) have demonstrated that the ingestion of large amounts of nicotinic acid to pellagrins is followed by an increase in the coenzyme I content of human erythrocytes and muscle. However, they found no bearing upon the coenzyme II content of the tissue. They also observed that the coenzyme I content of erythrocytes does not decrease significantly in varying stages of pellagra whereas the coenzyme I content of pellagrins muscle decrease as the deficiency becomes more severe.

Briggs et al (1943) reported that chicks receiving purified diets free of nicotinic acid show a marked lowering of the nicotinic acid and coenzyme I content of breast muscle. Anderson et al (1944) examined the effect of 3 different levels of nicotinic acid in a synthetic ration (basal, 1.5 mg niacin and 10 mg niacin/100 g ration) on the level of nicotinic acid and coenzyme I in different tissues of chick. They demonstrated that there is no increase in the weight of the chicks when the supplement is increased to 10 mg per 100 g ration, but there is a marked increase in nicotinic acid and coenzyme I content of breast and leg muscles. In the liver, however, the free nicotinic acid was the same for the basal and 1.5 mg supplemented groups, and doubled when the supplement was increased to 10 mg %, while the coenzyme I values remained approximately the same. Therefore, no such definite correlation between the level of nicotinic acid in a diet and that of coenzyme I in liver tissue is established. Furthermore, they observed that brain tissue is low in coenzyme I regardless of the level of nicotinic acid tested.

Singal et al. (1948b) measured the nicotinic acid levels in different tissues of rats on corn rations and found them to be subnormal in the brain, liver, and muscle of the deficient animals but normal in other tissues. Williams et al (1950) showed that NAD levels in rat liver fell to very low values when tryptophan and niacin were excluded from the diet. The addition of these two compounds to the diet restored the NAD levels to normal.

However, no such decrease was found in brain. Duncan and Sarett (1951) had shown, using human subjects, that blood pyridine nucleotides increased much more rapidly when niacin was ingested in large amounts than when tryptophan was ingested. Burch et al (1955) reported that rats fed low amounts of tryptophan and niacin had subnormal amounts of NAD and NADP in blood cells and liver. In 1956, Kaplan et al found that injection of large doses of nicotinamide elicited a 9- to 12-fold increase of NAD in the livers of mice. Under the same conditions, no change in the levels of this compound in brain was noted. Later, Burton et al (1958) injected nicotinamide and reserpine concomitantly to mice and observed that the two compounds acted synergistically to increase NAD synthesis in mouse liver. Tupule (1958) reported that the livers from rats fed a protein-deficient diet showed a lowering of the ratio of oxidized to reduced coenzymes. Both pyridine nucleotides were lowered, the oxidized forms more so than the reduced ones. Similarly, Spirtes and Alper (1961) studied the NAD and NADH levels in the livers of niacin-deficient and protein-deficient mice. They found a decrease of NAD and NADH in the liver of niacin-deficient adult mice and no change in the NAD/NADH ratio. On the other hand, protein-deficient mice developed a sharply lowered liver NAD content and an unchanged NADH level.

Garcia-Bunuel et al (1962) measured the levels of the pyridine nucleotides in liver and brain of rats made

deficient by a low-tryptophan and niacin-free diet. There was a significant fall in NAD (34%), NADH (18%), and NADP (13%) levels in the brain of deficient rats compared with controls. In the livers of niacin deficient rats the levels of NAD, NADH, NADP, and NADPH were, respectively, 34%, 59%, 73%, and 55% of normal controls. In contrast, NADPH levels in the brain were not affected by diet. Also Greengard et al (1968) found lower levels of NAD and NADP in the liver of rats treated with niacin-free and tryptophan-deficient diets.

In 1960, contrasting reports have been published by Morrison et al regarding the pyridine nucleotide contents in response to niacin deficiency. They determined the effects of an amino acid imbalance on growth and liver pyridine nucleotide concentrations of rats fed niacin-deficient diets containing 8% of casein. Although rats fed the niacin-deficient diet grew very little and apparently developed a niacin deficiency, their liver pyridine nucleotide concentrations were not depressed and a level (2.5 mg/100 g of diet) of niacin which prevented the deficiency condition did not increase pyridine nucleotide concentration. Similarly, Brown (1964) also reported that deficiencies of tryptophan or niacin in a diet had little or no effects on the levels of NAD in rat brain. Even though the inanition was severe, and the animals were near death, the NAD levels in brain were remarkably unchanged. He suggested that in brain, the turnover of NAD is almost nil, or that the levels

are maintained at the expense of other tissues, such as the liver, which are depleted during deficiency. According to Ichiyama et al's reports, the amount of total NAD in diabetic rat livers was maintained at a normal level (1967).

3. PROTEIN TURNOVER

A) Role of Coenzyme in Protein Turnover

A number of examples are known in mammalian cells where the rate of protein catabolism changes in response to supply of substrates, coenzymes, or other factors that bind to the polypeptide. Interactions with small molecules can alter a number of other chemical and physical properties of a polypeptide, including its sensitivity to proteolytic enzymes (Citri, 1973). It has long been recognized that administration of tryptophan or α-methyltryptophan to a rat leads to increased levels of tryptophan pyrrolase as a result of decreased rates of enzyme degradation as well as increased rates of enzyme synthesis (Schimke et al 1965, Pirias et al 1967).

In addition, coenzymes have been found to stabilize protein conformations in vitro in a fashion that may influence intracellular degradative rates. Bond (1971) reported that NAD addition to the incubation mixture fully protected glyceraldehyde-phosphate dehydrogenase from inactivation by all of the proteases (chymotrypsin, subtilisin and pronase) and partially protected α-glycero-phosphate and lactate dehydrogenases. He also observed that pyridoxal phosphate reduces the sensitivity of serine dehydratase and tyrosine aminotransferase to trypsin and chymotrypsin. Perhaps of greater physiological relevance

is the recent finding by Katunuma and co-workers (1973) of proteases that can inactivate selectively pyridoxal-requiring enzymes and proteases for NAD-requiring enzymes. The sensitivity of several such enzymes to these group-specific proteases increases upon removal of their respective cofactors. The greater sensitivity of the apoenzymes to such proteases can explain the low levels of vitamin-requiring enzymes in vitamin B_6 and niacin deficiency or the decreased degradation of serine dehydratase upon administration of pyridoxine. They suggested that the conversion of holo- into apo- form is the rate-limiting and initiating step in the degradation of these B_6 enzymes in vivo.

Litwack and co-workers (1973) proposed that the tightness of coenzyme binding could be a major determinant of degradative rates in vivo. They pointed out that several liver pyridoxal enzymes which are inducible by glucocorticoids have short half-lives and dissociable coenzymes, while enzymes which are poorly inducible by glucocorticoids have long half-lives and nondissociating coenzymes. Moreover, these workers showed that the relative rate of dissociation of the coenzyme roughly correlated with the half-life of five liver enzymes. Thus they suggested that the rate of coenzyme dissociation may be a rate-limiting step in enzyme degradation in vivo. In contrast, Clark and Fuller (1976) have found that the rate of dissociation of coenzymes does not change appreciably during a period when the activity

half-life of ornithine decarboxylase decreases 2-6 fold, indicating there is no close link between the two. To test whether pyridoxal phosphate-dependent enzymes are stabilized by a high concentration of pyridoxal phosphate *in vivo*, Hunter and Harper (1976) have measured rates of decline of the activities of three pyridoxal phosphate-dependent enzymes (serine dehydratase, ornithine aminotransferase and tyrosine aminotransferase) in liver from rats. They noted that vitamin B_6 deficiency affects different enzymes in different ways. Serine dehydratase activity declined more rapidly in vitamin B_6 deficient than in control liver; however, ornithine aminotransferase and tyrosine aminotransferase activities were equally stable in deficient and control liver. Ornithine aminotransferase ($t_{½}$ = 1.3 days) was predominantly in holoenzyme form in both control and deficient rats, whereas tyrosine aminotransferase ($t_{½}$ = 4.4 hrs) was predominantly in apoenzyme form in both groups. The proportion of serine dehydratase ($t_{½}$ = 0.9 - 1.2 days) in apoenzyme form was twice as great in deficient as in control liver. Therefore, the absolute rate of decay of activity seems to be determined by some intrinsic property of the enzyme other than the rate of dissociation into coenzyme and apoenzyme.

Very recently, Lee *et al* (1977) employed immunochemical analysis to determine not only the decay of enzyme activity, but also synthesis and degradation of the tyrosine aminotransferase and alanine aminotransferase in

B_6-deficient rats. The authors found that coenzyme deficiency results in a significant reduction in tyrosine aminotransferase levels, a reduction clearly attributable to reduced synthesis of enzyme. The degradation rate of the actual enzyme molecules, measured by immune precipitation after labeling, however, was identical for deficient and control enzymes ($t_{\frac{1}{2}}$ = 1.5 hrs). In contrast to the results with tyrosine aminotransferase, the rate of synthesis of alanine aminotransferase was not significantly changed in B_6 deficiency. They noted that the inactive apo-form enzyme is synthesized and degraded at the same rate as the enzymatically active enzyme of the B_6-fed control rats, thus accounting for equal synthesis and degradation rates, but lower enzyme activity in deficiency. They pointed out that coenzyme interaction with enzymes "was not a significant determinant in intracellular degradation" of the enzymes, since the more dissociated pyridoxal phosphate-deficient tyrosine aminotransferase enzyme was degraded at the same rate as the undissociated holo-enzyme of the control rat. They also believe that coenzyme binding and rate of degradation, although not causally related, are both reflections of structural properties of the enzyme which determine coenzyme binding and degradation rate. Hence, they proposed that tyrosine aminotransferase has a highly flexible structure, resulting in ready coenzyme dissociation at the same time as rapid degradation; alanine aminotransferase presents a more hydrophobic structure which binds pyridoxal phosphate

tightly and also in less readily susceptible to attack by degradative enzymes. Furthermore, they speculated that the prolonged B_6 deficiency may cause an endocrine dysfunction which may indirectly affect the normal hormonal regulation of enzyme synthesis, at least as far as tyrosine aminotransferase is concerned.

B) Lysosomes

The possibility that the lysosomal system is involved in proteolysis is suggested by the localization of cathepsins within lysosomes (Barrett, 1972) and by the fact that isolated lysosomal proteases are capable of degrading large proteins to free amino acids or small peptides (Coffey and Duve, 1968). Moreover, it is known that lysosomes are capable of digesting exogenous proteins after its endocytic uptake by liver (Mego et al 1967). Jacques (1969) has proposed a broad discriminatory capacity for uptake into digestive vacuoles based upon a putative variability among proteins in their affinity for the hydrophobic surface of membranes. Evidence in this direction has been reported by Dean (1975), who notes that proteins that turn over more rapidly in vivo have a greater absorbability to lysosomes than those that turn over more slowly. However, Huisman et al. (1974b) in similar experiments could find no such selectivity. Recently Ballard (1977) has suggested that both lysosomal and non-lysosomal pathways of protein degradation are operative, but with different specificities,

and that short-lived proteins (abnormal proteins) are preferentially degraded by non-lysosomal pathways.

Segal et al (1976) and Bohley et al (1972) reported analogous studies with lysosomal proteases at acid pH. Upon incubation with lysosomal enzymes, liver proteins with short half-lives in vivo tended to be degraded preferentially. In addition, lysosomal cathepsins selectively hydrolyze analog-containing proteins from mammalian cells. Such results are consistent with an involvement of lysosomal enzymes in the degradation of cell proteins and the possibility that the rate-limiting step in proteolysis occurs within this organelle. They certainly do not prove such conclusions, since analogous results have been obtained with many non-lysosomal proteases at neutral pH.

C) Size of Proteins

During the course of studies on the heterogeneity of turnover of proteins of plasma membrane and endoplasmic reticulum, Dehlinger and Schimke (1970) made the observation that relative rates of protein turnover, as measured by the double-isotope method of Arias et al (1969), were related to the size of the protein subunit as electrophoresed on SDS gels. Dice and Schimke (1972) have also found this same correlation for proteins of rat liver ribosomes. Such studies have led these workers to propose that the correlation of size and rate of degradation is based on the overall greater chance of a larger protein being "hit" by a protease, producing an initial rate-limiting peptide bond

cleavage.

Larger protein components of certain organelles also appear to be degraded more rapidly than the smaller proteins. Correlations between size and degradative rates have been demonstrated for the protein subunits of the endoplasmic reticulum and plasma membrane from rat and mouse liver (Gurd et al 1973), for plasma membrane proteins from cultured baby hamster kidney cells, and for membrane proteins in brush border of rat intestine (Alpers, 1972). A similar correlation between size and degradation rate was also observed among the protein components of chromosomes (Dice and Schimke, 1973) and the multienzyme complex, fatty acid synthetase (Tweto et al, 1972). Dice and Goldberg (1975) found a correlation between the logarithm of the subunit molecular weight and the measured or estimated half-life of 33 rat liver proteins. A straight line plot of the log of the subunit molecular weight versus the turnover time showed a coefficient of correlation of -0.60.

D) Charge

Recently Dice and Goldberg (1975) reported a correlation between degradative rates in vivo and isoelectric points among soluble proteins of rat liver, skelatal muscle, kidney and brain. In a wide variety of mammalian tissues, proteins with low isoelectric points tend to be degraded faster than those with neutral or basic isoelectric points. In addition, a highly significant relationship (γ= -0.824,

$P<0.01$) was demonstrated by statistical analysis of 23 liver proteins. These statistical studies, as well as direct experiments, indicated that protein size and charge are independent factors influencing half-life. Thus proteins with a given isoelectric point still show a correlation between molecular weight and intracellular half-life. These results predict that particularly stable proteins should be both small and basic. In fact the histones, which fit these criteria exactly, are probably the most stable polypeptides in the cell (Dice and Schimke, 1973). In agreement with the findings of Dice and Goldberg (1975), Duncan and Bond (1977) presented evidence that acidic proteins from liver cytosal have larger subunits than do basic proteins.

Momany et al (1976) calculated a parameter which relates the amino acid composition and subunit size of a protein to the degradative rate in vivo. A plot of this parameter versus the half-life of 11 rat liver proteins is linear with a coefficient of correlation of -0.96. They proposed that the density of excess acidic amino acids on the surface of the protein is the most important factor in determining differential turnover. Recent observations by Dice et al (1978) indicate that the tendency of acidic proteins to turn over more rapidly is decreased or abolished in liver and muscle of starved or diabetic animals. Thus the features of the degradative process that are responsible for the correlation between net charge and half-life must be masked or altered during the enhanced protein degradation

seen in diabetes and starvation. They also reported that glycoproteins tend to be degraded more rapidly than non-glycoproteins. Very recently Dice et al (1979) examined further the relationship between protein net charge and catabolic rate of soluble proteins from rat lung, heart, and testes, and from human fibroblasts and mouse-embryo cells grown in culture. They indicated that the more rapid degradation of acidic proteins is a general characteristic of degradation of soluble proteins in mammalian tissues.

E) Denaturation

It has been suggested that the rate-limiting step in degradation of most cell proteins is spontaneous denaturation (Li and Knox, 1972). The actual free-energy difference between the native conformations of most globular protein and various unfolded states is surprisingly small (Pace, 1975). Free energies of denaturation of enzymes are of the order of only 5-15 kcal $mole^{-1}$ compared to 80 kcal $mole^{-1}$ for the dissociation energy of an aliphatic carbon-carbon bond. Thus, it has been suggested that denaturation may be a relatively frequent event under in vivo conditions. A study of the stability of a number of liver enzymes by Hopgood and Ballard (1974) indicated that most enzymes lose activity in homogenates at rates that correlate roughly with their in vivo half-lives. It has been speculated that enzyme inactivation precedes or can occur in the absence of proteolytic cleavage. In fact, Ballard et al (1974) have

shown that the loss of PEP carboxykinase activity in homogenates involves initial denaturation, precipitation, and subsequently limited proteolytic cleavage. It is noteworthy that inactivation of this enzyme was more rapid in the presence of microsomal fractions than lysosomal fractions.

Bond (1975) has studied a series of liver enzymes in vitro and showed that their half-lives in vivo correlate roughly with rates of temperature inactivation and inactivation at pH 5.0. She suggested that acid inactivation within lysosome rather than proteolytic attack may be the rate-limiting step in the degradative process. Nevertheless, these findings by Bond may indicate that the ease of denaturation is a feature of most proteins with short half-lives. Bohley and colleagues (1976) observed that proteins with short half-lives tend to precipitate out of solution more readily than stable cell components.

F) Covalent Modification

The observations that approximately 80% of the intracellular population of Ehrlich ascites cell (Brown and Roberts, 1976) and other mammalian proteins are α-N-acetylated suggests that this blocking group serves an important biological function. Jörnvall (1975) demonstrated that α-N-acetyl groups may protect proteins from proteolytic degradation. Recently Brown and Roberts (1976) also reported that their preliminary experiments with L-cells suggest a lower turnover

rate for proteins containing acetylated amino-terminal residues than for those with free amino-terminals. Furthermore, Dice and Goldberg (1975b) reported that acetylation and isoelectric point are important in determining the *in vivo* protein half-life. On the other hand, Roberts and Yuan (1975) have provided evidence that proteins acetylated with acetic anhydride in tissue culture have the same half-life as proteins that are not acetylated. In support of the findings of Roberts and Yuan (1975), Mauk *et al* (1976) investigated the significance of amino terminal acetylation in the turnover of Hb by measuring the synthesis and degradation of HbA and HbB in domestic cat blood. Their results showed that the turnover of Hb is unrelated to isoelectric point or to amino-terminal acetylation of the protein. Recent observations by Brown (1979) demonstrated that α-N-acetylated L-cell proteins are not unusually stable and suggest that α-N-acetylation does not protect proteins from proteolytic degradation.

In a series of reports, Carlson and Kim (1974a and b) provided evidence that rat liver acetyl-CoA carboxylase is controlled by covalent modification: phosphorylation results in inactivation of the enzyme, whereas dephosphorylation results in activation. Examination of the sedimentation behavior of treated acetyl-CoA carboxylase revealed that phosphorylation of rat liver enzyme is accompanied by depolymerization and inactivation of the enzyme. In support of covalent modification as a mode of control of acetyl-CoA

carboxylase *in vivo*, they have shown that epinephrine treatment of isolated epididymal fat tissue causes depolymerization of carboxylase (Lee and Kim, 1978).

G) Degradation of Abnormal Proteins

The first evidence for rapid intracellular degradation of abnormal proteins was obtained by Rabinowitz and Fisher (1961) with rabbit reticulocytes incubated with amino acid analogs. These workers showed that the incorporation of t-α-amino-β-chlorobutyric acid in place of valine and S-(β-amino-ethyl)-cysteine in place of lysine into Hb by reticulocytes leads to rapid catabolism of this protein.

Recently Etlinger and Goldberg (1976) have observed that the degradation of analog-containing globin in reticulocytes is inhibited by dinitrophenol and various sulfhydryl-blocking reagents, but not by inhibitors of protein synthesis. In addition, incorporation of canavanine by cultured human fibroblasts and baby hamster kidney cells promotes protein degradation significantly (Bradley *et al*, 1976). The effects of analogs on protein turnover in Reuber H35 hepatoma have been extensively studied by Knowles *et al* (1975). Canavanine promoted the degradation of average cell protein several fold as well as the degradation of PEP carboxykinase. On the other hand, Johnson and Kenny (1973) have studied the incorporation of several tryptophan analogs on the half-life of tyrosine aminotransferase in rat hepatoma cells; although the enzyme became

more sensitive to high temperatures, its half-life *in vivo* was not shortened.

H) Energy Requirement For Degradation

The early study of Simpson (1953) demonstrated an inhibition of the degradation of proteins in rat liver by anaerobiosis or by the addition of cyanide or dinitrophenol. The degradation of both the labile cell proteins and the more stable components in rat fibroblasts (Poole, 1973) and in *E. coli* (Goldberg *et al*, 1975) show such a dependence on metabolic energy.

Several workers have suggested an important role of ATP in the functioning of lysosomes. de Duve and Wattiaux (1966) indicated that ATP facilitates the entry of protein into lysosomes either through some specific uptake process or possibly through some continuous "vacuuming of the cytoplasm" by the lysosomal apparatus. Similarly, Hayashi *et al* (1973) reported that degradation of Hb by isolated lysosomes is stimulated by ATP at pH 4.5 and concluded that ATP facilitated Hb transport into these structures. However, Huisman *et al.*(1974a) showed that ATP under these conditions promoted lysosomal rupture and probably the subsequent extralysosomal degradation of the Hb. Recent studies reported by Hershko and Tomkins (1971) indicated that the degradation of tyrosine aminotransferase in hepatoma cell culture was dependent on ATP. Following induction with dexamethasone, tyrosine aminotransferase activity usually returned rapidly

to the preinduction level. However, the addition of NaF or kF remarkably inhibited the degradation of both induced tyrosine aminotransferase and general cell proteins.

Mego and co-workers (1972) have found that ATP may be required for the maintenance of an acid pH within lysosomes. They also noted that hydrolysis of pinocytized [^{125}I]albumin in liver slices, which presumably occurs within secondary lysosomes, can be inhibited by dinitrophenol, or azide. In addition, they showed that albumin degradation in crude preparations of kidney lysosomes increased upon addition of ATP under alkaline conditions but not at pH 5.0 or below. These findings were interpreted to imply that an energy-dependent proton pump is responsible for the low pH within the lysosome.

(I) Hormone

Knox and associates (1951) demonstrated that the hepatic enzyme tryptophan pyrrolase could be markedly increased in activity following treatment of rats with cortisone. It was shown that the rate of synthesis of tyrosine aminotransferase is specifically enhanced by hydrocortisone (Kenney, 1962), insulin and glucagon (Holten and Kenney, 1967). Degradation of tyrosine aminotransferase is inhibited by agents that block protein synthesis, such as cycloheximide or puromycin (Grossman and Maurides, 1967), and by certain inhibitors of RNA synthesis (Lee et al, 1970).

Several workers (Manchester et al, 1959; Wool and Weinshelbaum, 1960; Shimizu and Kaplan; 1964), using isolated diaphragms, have shown that there was a reduction in the incorporation of labelled amino acids (glycine, histidine, phenylalanine and methionine) into muscle protein in rats which were given cortisone or cortisol. The increased loss of labelled protein from muscle of cortisone-treated rats by Goldberg (1969b) was taken to show that cortisone increased protein catabolism. Studies in vivo (Goldberg, 1969a and with isolated muscle from hormone-treated rats indicate that growth hormone induces growth by stimulating protein synthesis without changing degradation. Thyroidectomy or hypophysectomy, either of which greatly diminishes thyroid hormone production, decreased protein degradation in skeletal muscles of rats (Griffin and Goldberg, 1978). Physiologic doses of thyroxine or triiodothyronine induced muscle growth and increased both protein synthesis and protein degradation. In addition, thyroid hormones were found to increase protein degradation in liver but not in heart or kidney.

Mortimore and co-workers (1970) demonstrated that insulin reduces protein catabolism in perfused liver. The reduction in proteolysis was associated with decreased development of large, osmotically sensitive lysosomes, which appeared during perfusion in the absence of the hormone (Neely et al, 1974). Wildenthal (1973) found that insulin reduced the activity of cathepsin D in fetal mouse hearts in organ culture. The hormone also has been reported to reduce

protein degradation in other tissues including adipose tissue (Minemura et al, 1970), fibroblasts (Hershko et al, 1971), and skeletal muscle (Goldberg et al, 1974). In addition, this hormone stimulates protein synthesis and transport of amino acids and glucose in many tissues (Fulks et al, 1975).

Glucagon probably stimulates net liver protein catabolism by promoting autophagocytosis. This process involves the subcellular redistribution of lysosomal enzymes from "substrate-poor" primary lysosomes to "substrate-rich" autophagic vacuoles (Deter, 1971). This overall effect is manifested by increased urea formation (Miller, 1960), a reduction of liver protein content (Miller, 1965) and an increase in the net release of amino acids from protein (Mallette et al, 1969).

J) Deprivation of Nutrients

In general, starvation increases the activities of a number of enzymes involved in gluconeogenesis, amino acid catabolism and lipid catabolism. These changes involve an increase in the activity of glucose-6-phosphatase (Harper, 1959). PEP-carboxykinase (Nordie et al, 1965), glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase (Fitch and Chaikoff, 1961), serine dehydratase and tyrosine transaminase (Goswami et al, 1966). However, it appears that the activities of phosphorylase (Gutman and Shafrir, 1964), phosphoglucomutase, phosphohexose-isomerase,

malic enzyme and glucose-6-phosphate dehydrogenase (Weber and Cantero, 1957), citrate cleavage enzyme (Kornacker and Lowenstein, 1963), and β-hydroxy-β-methylglutaryl-CoA reductase (Regen et al, 1966) decrease.

It has been known that food deprivation remarkably reduces protein synthesis and RNA content in muscle (Millward et al, 1973). The isotopic experiments of Millward (1970) and Gan and Jeffay (1971) first showed accelerated protein breakdown of skeletal muscle during fasting. Li and Goldberg (1976) studies muscle from fasting rats in vitro and observed increased degradation in the "pale" muscles but not in the "dark" ones. The relative importance of increased proteolysis and decreased synthesis in the mobilization of muscle protein remains uncertain (Garlick et al, 1975). In rat muscle, protein synthesis decreases one day after food deprivation, while increased degradation is first evident the subsequent day (Li and Goldberg, 1976). In fasted animals, accelerated proteolysis is more pronounced in liver than in muscle (Garlick et al, 1975). In starvation, synthesis of rRNA of rat livers decreases rapidly and reaches a minimum after 24 hrs starvation (Rickwood and Klemperer, 1971). This decrease is correlated with a decrease in activity of the nucleolar RNA polymerase A which catalyzes synthesis of rRNA. A second RNA polymerase B is concerned with synthesis of mRNA (Chambon, 1971). This polymerase is less rapidly affected by starvation. Such a difference in the response of rRNA and mRNA to fasting implies

that their synthesis is regulated by separate mechanisms.

In studies of intact animals, Henshaw et al (1971) observed that fasting involves a lower protein synthesis rate per microgram of muscle RNA in addition to a fall in tissue RNA. Furthermore, they found not only a decreased ribosome content but also a lower polysome activity in livers of fasted rats.

Buse and Reid (1975) proposed the hypothesis that leucine inhibits protein degradation and promotes protein synthesis in muscle. Therefore, it has been suggested that leucine may act as a regulator of the turnover of protein in muscle cells. Goldberg and Odessey (1972) reported a threefold acceleration of the oxidation of branched-chain amino acids by isolated muscles obtained from rats after a 67 hr fast. The branched-chain amino acids are the only amino acids that are not concentrated by muscle cells to a significant degree (Bergström et al, 1974). The oxidation of leucine may provide appreciable ATP to the muscle in starvation (Chang and Goldberg, 1978a) and leads to decreased oxidation of glucose (Chang and Goldberg, 1978b).

GENERAL EXPERIMENTAL PROCEDURES

1. Animals

One-day old Japanese quail (Coturnix japonica) were obtained from the University hatchery. They were housed for all experiments, in electrically-heated, thermostatically-controlled batteries with raised wire floors. The birds had free access to water at all times and were exposed to constant lighting.

2. Diets

To produce niacin deficiency, a niacin free diet was fed ad libitum (Briggs et al., 1942). The composition of the experimental diet which was purchased from U.S. Biochemical Corporation is given in Table 1. A microbiological assay of this basal diet showed a nicotinic acid content of 0.3 mg per 100 g of diet (Briggs et al., 1942). Some birds were fed a nicotinic acid supplemented diet. The composition of this diet was identical to that of the niacin free diet except that nicotinic acid was added. Chick starter diet containing 21% protein was obtained from a commercial source, and used briefly on newly hatched chicks.

Table 1. Percent Composition of Experimental Diets.

Ingredients	Niacin deficient diet	Niacin supplemented diet
	%	%
Vitamin free casein[1]	18.0	18.0
Dextrin	61.0	61.0
Gelatin	10.0	10.0
L-Cysteine	0.3	0.3
Cod liver oil	3.0	3.0
Soybean oil	5.0	5.0
Monocalcium phosphate	1.0	1.0
Vitamin mix[2]	2.2	2.2
Phillips Hart salt mix[3]	5.0	5.0
Nicotinic acid	—	0.007

[1]The amino acid composition (% of diet) was as follows: L-lysine, 1.43; L-histidine,0.45; L-arginine, 1.19; L-aspartic acid, 2.3; L-threonine, 0.83; L-serine, 1.42; L-glutamic acid, 4.24; L-proline, 2.58; L-glycine, 2.62; L-alanine, 1.31; L-cystine, 0.40; L-valine, 1.16; L-methionine, 0.58; L-isoleucine, 0.83; L-leucine, 1.59; L-tryptophan, 0.19; L-tyrosine, 0.69; and L-phenylalanine, 0.86.

[2]The vitamin mix per kg of diet consisted of the following composition: ascorbic acid, 980 mg; biotin, 0.44 mg; folic acid, 1.98 mg; pyridoxine, 22 mg; riboflavin, 22 mg; thiamine, 22 mg; p-aminobenzoic acid, 110 mg; choline chloride, 1.650 mg; inositol, 110 mg; menadione, 49.5 mg; D-calcium pantothenate, 66 mg; vitamin B_{12}, 0.03 mg; calciferol (D_2), 2,197 IU; vitamin A acetate, 19,780 IU; and α-tocopherol, 109 IU.

[3]The composition of the mineral mix (mg/kg of diet) was as follows: K_2HPO_4, 16,130 mg; $CaCO_3$, 15,000 mg; NaCl, 8,440 mg; $MgSO_4$, 5,130 mg; $CaHPO_4.2H_2O$, 3,750 mg; ferric citrate, 1,380 mg; KI, 40 mg; $ZnCl_2$, 15 mg; $CoCl_2.6H_2O$, 22 mg; and $MnSO_4.H_2O$, 250 mg.

3. Chemicals

The following chemicals were used: Rabbit muscle aldolase, (EC. 4.1.2.13) α-glyceraldehyde-3-phosphate dehydrogenase, (EC. 1.2.1.12) α-glycerophosphate dehydrogenase, (EC. 1.1.1.18) fructose-1,6,-diphosphatase, (EC. 3.1.3.11) phosphoglucose isomerase, (EC. 5.3.1.9) triosephosphate isomerase, (EC.5.3.1.1) lactic dehydrogenase, chicken liver malic enzyme, (EC.1.1.1.27) bovine liver glutamic dehydrogenase, (EC.1.1.1.40) yeast glucose-6-phosphate dehydrogenase, (EC.1.4.1.2) alcohol dehydrogenase, (EC.1.1.1.49) 3-phosphoglycerate kinase, (EC.1.1.1.1) NAD, NADH, NADP, NADPH, (EC.2.7.2.3) FDP, ATP, dihydroxyacetone phosphate, 2-oxoglutarate, glycerate-3-phosphate, glucose-6-phosphate, malic acid, pyruvic acid, ascorbic acid, tris, methemoglobin, bovine serum albumin, conalbumin, ovalbumin, cytochrome C, dextran blue, tryptophan, leucine, isoleucine, 6-aminonicotinamide, and phenylmethylsulfonyl fluoride were purchased from the Sigma Chemical Co., St. Louis, Mo. [Carboxy-^{14}C] Nicotinic acid (50-60 mCi/mmole), L-[4,5-^{3}H]-leucine (100 Ci/mmole), L-[U-^{14}C]-leucine (300 mCi/mmole), PPO(2,5-diphenyloxazole), POPOP (p-bis-[2-(5-phenyloxazolyl)] benzene); NCS (tissue solubilizer), Triton X-100 (Scintillation grade) were from Amersham/Searle. Other chemicals purchased were ammonium persulfate, toluene (scintillation grade), glacial acetic acid, (Fisher Scientific Co.), phenol reagent (Harleco), DEAE (Whatman), Sephadex G-200 (Pharmacia Fine Chemicals), Ampholine^{R}, pH 3-10, (LKB), and acrylamide, bisacrylamide, riboflavin, and coomassie brilliant blue (Bio. Rad Lab).

4. Measurements of Pyridine Nucleotides

a) Extraction and determination of NAD and NADP

An adequate portion of tissue was weighed for the extraction and determination of NAD and NADP. Tissue samples and 5 ml of cold 0.6 N $HclO_4$ were introduced into centrifuge tubes, homogenized for 1.0 minute in an ice box using a Polytron homogenizer (Kinematica, GmbH) and the homogenate was then centrifuged at 5000 x g for 5 minutes in a Sorvall RC 2B centrifuge. The pH of the supernatant was adjusted to 7.2 to 7.4 by the addition of 3N KOH along with 0.2 ml of 1M K_2HPO_4. The pH adjusted extract was recentrifuged at 5000 x g for 5 minutes and the clear supernatant was collected for immediate analysis.

Enzymatic assays of NAD and NADP were carried out according to the procedure of Klingenberg (1974). NAD and NADP were determined by quantitative reduction to NADH and NADPH respectively using alcohol dehydrogenase (ADH) and glucose-6-phosphate dehydrogenase (g6p-dH) enzyme systems according to reactions given below.

$$\text{Ethanol} + \text{NAD} \underset{\leftarrow}{\overset{\text{ADH}}{\rightarrow}} \text{acetaldehyde} + \text{NADH} + H^+$$

$$\text{Glucose-6-phosphate} + \text{NADP} \underset{\leftarrow}{\overset{\text{g6P-dH}}{\rightarrow}} \text{6-phosphogluconate} + \text{NADPH} + H^+$$

The assays were performed at 340 nm at 30°C in 1-cm cuvettes in a Gilford Model 2400 spectrophotometer using 0.1 or 0.5 absorbance as a full scale. Duplicate analyses were performed routinely on the extracts. The millimolar extinction coefficient of reduced coenzymes was taken to be 6.22×10^6 cm^2/mole.

For a typical NAD determination, 1 ml of clear extract was incubated with 1 ml of 0.1 M pyrophosphate buffer (pH 8.8) and 0.05 ml of absolute ethanol for 15 minutes at 30°C. After incubation the change in absorbance of the reaction mixture was followed at 340 nm for approximately 10 to 15 minutes until a constant value was reached (A_1). Finally, 2 units of alcohol dehydrogenase was pipetted into a cuvette and the change in absorbance at 340 nm was followed for approximately 10 minutes until a second constant reading was obtained (A_2). The final reading (A_2) minus the initial reading (A_1) was used to calculate the levels of NAD present in the extract.

For the determination of NADP, the same procedure was followed as in the case of NAD. The assay mixture consisted of 0.1 ml of 0.2 M glucose-6-phosphate, 0.02 ml of 1 M $MgSO_4 \cdot 7H_2O$, and 2.5 ml of sample extract. After incubation at 30°C for 15 minutes, the change in absorbance at 340 nm was followed for 10 to 15 minutes until a constant reading was obtained (A_1). Then 10 units of glucose-6-phosphate dehydrogenase (300 units/mg protein) was added to the assay mixture and the subsequent absorbance change was followed until the reaction was complete (A_2). As for NAD, the absorbance change ($\Delta A = A_2 - A_1$) was used in the calculations for concentration of NADP in extracts.

b) Extraction and determination of NADH and NADPH

An adequate portion of tissues was weighed for the measurements of NADH and NADPH. The sample tissues were homogenized in 5 ml of cold alcoholic 0.5 N KOH containing 1 mM 2-mercaptoethanol with a Polytron homogenizer for 1.0 minute . The homogenate was heated in a 90°C water bath for 5 minutes. Immediately following heating, the sample was placed in an ice box for 5 minutes. The pH of extract was then brought to 7.8 by the addition of an equimolar mixture of 0.5 M triethanolamine-phosphate buffer (pH 5.8). Finally the extract was centrifuged at 30,000 x g for 5 minutes and filtered through Millipore filter paper (type HA 0.45 um).

NADH and NADPH were also determined in the extract by Klingenberg's method (1974). The following reactions are involved:

$$\text{Dihydroxyacetone phosphate} + \text{NADH} + H^+ \rightleftharpoons \text{glycerol-3-phosphate} + \text{NAD} \quad (1)$$

$$\text{2-Oxoglutarate} + NH_4^+ + \text{NADPH} \rightleftharpoons \text{glutamate} + \text{NADP} \quad (2)$$

The measurement of NADH is based on the oxidation of NADH by dihydroxyacetone phosphate and glycerol-3-phosphate dehydrogenase (equation (1)). Likewise, the measurement of NADPH is based on the oxidation of NADPH by 2-oxoglutarate and glutamic dehydrogenase (equation (2)). For the determination of NADPH, the oxidation of NADPH must be preceded by that of NADH.

Two milliliters of clear extract were incubated with 0.05 ml of substrate mixture containing 0.5 mM dihydroxyacetone phosphate, 2.5 mM 2-oxoglutarate, and 5 mM NH_4^+ for 15 minutes at 30°C. After incubation the change in absorbance of the reaction mixture was followed at 340 nm for 10 to 15 minutes until a constant reading was attained (A_1). Then 9 units of glycerol-3-phosphate dehydrogenase (175 units/mg protein) was added to the reaction mixture and the absorbance change (A_2) was followed for an additional 10 to 15 minutes until the reaction was complete. The difference in absorbance change between A_2 and A_1 was used to determine the NADH content. In order to measure NADPH, 10 units of glutamic dehydrogenase (50 units/mg protein) was subsequently added to the reaction mixture and the absorbance change was followed for an additional 10 to 15 minutes (A_3). The absorbance change thus obtained ($\Delta A = A_3 - A_2$) was used to calculate the NADPH content.

5. Enzyme assays

For all enzyme assays, except for tryptophan pyrrolase, tissue samples were homogenized with a Polytron homogenizer in 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA and 5 mM 2-mercaptoethanol. Samples for tryptophan pyrrolase assay were homogenized in cold 20 mM potassium phosphate buffer (pH 7.0) containing 0.14 KCl. The homogenates were centrifuged at 50,000 x g for 15 minutes and resulting supernatants were incubated with cocktail reaction mixtures at 30°C for 15 minutes prior to the initiation of the enzyme reactions. All assays were monitored with a Gilford Model 2400 recording spectrophotometer. Each reaction except for tryptophan

pyrrolase was followed at 340 nm for 10 minutes and the initial linear portion of the curve was taken as the rate of reaction. Also, duplicate or triplicate assays were performed routinely on the enzyme extracts. The molar extinction coefficients for the aldolase reaction was 12.44×10^6 cm^2/mole and 6.22×10^6 cm^2/mole for the remaining five enzymes except tryptophan pyrrolase. All enzyme activities were expressed in terms of a unit, defined as the amount of enzyme that catalyzes the formation of 1 µmole of product per minute or hour at 30°C.

Specific assays were as outlined below:

a) Aldolase (EC.4.1.2.13)

The aldolase assay was that reported by Marquardt (1969). Each cuvette contained 2.3 ml of the reaction cocktail which consisted of 50 mM Tris, 5 mM EDTA (pH 7.5), 0.2 mM NADH, 15 µg rabbit muscle glycerol-3-phosphate dehydrogenase, 15 µg triose-phosphate isomerase plus 0.01 ml of extract. The reaction was initiated by the addition of 0.025 ml of 0.48 M FDP (fructose-1, 6-diphosphate) to the cuvette.

b) Fructose-1, 6-diphosphatase (EC.3.1.3.11)

Fructose-1, 6-diphosphatase activity was determined spectrophotometrically by following NADPH formation at 340 nm (Olson and Marquardt, 1972). The usual assay system contained 2.0 ml of 50 mM Tris, 1.0 mM EDTA (pH 7.5), 15 mM $MgCl_2$, 0.2 mM NADP, 10 µg phosphohexose isomerase, 10 µg glucose-6-phosphate

dehydrogenase plus 20 µl of the extract. The reaction mixture was incubated at 30°C for 30 minutes prior to the initiation of reaction by addition of 0.02 ml of 8.0 mM FDP. The long incubation period provided sufficient time to completely activate the enzyme (Marquardt's personal communication).

c) Glyceraldehyde-3-phosphate dehydrogenase (EC.1.2.1.12)

This assay was a modification of that reported by Velick (1955). Each cuvette contained 2.5 ml of 6.7 mM EDTA, 26.7 mM sodium pyrophosphate, 3.4 mM sodium arsenate, 3 mM 2-mercaptoethanol (pH 8.4), 0.33 mM NAD, 2 mM FDP, 15 µg aldolase and 15 µg triosephosphate dehydrogenase. The reaction was initiated by addition of 0.05 ml of the appropriate dilution of the enzyme extract.

d) Malic enzyme (EC.1.1.1.40)

This assay was a modification of that reported by Wise and Rall (1964). The assay mixture contained 2.5 ml of 54 mM Tris (pH 7.4), 5 mM $MnCl_2.4H_20$, 0.4 mM NADP and 0.05 ml of the appropriate dilution of the enzyme extract. The reaction was started by the addition of 0.1 ml of 15 mM L-malate.

e) Lactic dehydrogenase (EC.1.1.1.27)

Lactic dehydrogenase assay was a modification of that reported by Hirota et al., (1976). The assay mixture contained

2.5 ml of an equimolar mixture of Tris-acetate buffer (pH 7.5), 8 mM pyruvate, 0.08 mM NADH and 0.38 ml of distilled water. The reaction was initiated by the addition of 0.05 ml of the appropriate dilution of the enzyme extract.

f) Glutamic dehydrogenase (EC.1.4.1.3)

Glutamic dehydrogenase was a slight modification of that reported by Colman and Frieden (1966). The assay mixture contained 2.5 ml of an equimolar mixture of 10 mM Tris-acetate, 0.01 mM EDTA (pH 8.0), 50 mM NH_4Cl, 2.4 mM 2-oxoglutarate, and 1.0 mM NADPH. The enzyme reaction was started by the addition of the appropriate dilution of the enzyme extract.

g) Tryptophan pyrrolase (EC.1.13.11.11)

Tryptophan pyrrolase activity was measured by the method of Knox et al. (1955a). This method consists of an initial activation (conversion of the oxidized holoenzyme to the reduced holoenzyme) in a prior incubation followed by the measurement of the catalytic reaction. Liver tissue was homogenized in cold 0.02 M potassium phosphate buffer (pH 7.0) containing 0.14 M KCl using a Polytron homogenizer for 1.0 minute . The supernatant from a 25% liver homogenate obtained by centrifugation at 100,000 x g was preincubated at 37°C in small test tubes for 40 minutes (with gentle shaking) with the mixture containing 2 mg of methemoglobin/ml, 5 mM L-tryptophan, and 0.03 M freshly neutralized ascorbate. The following compounds were then added at 30°C to a 1 cm cuvettes: 0.7 ml of 0.2 M sodium phosphate

buffer (pH 7.0), 0.2 ml of 0.05 M L-tryptophan, 0.4 ml of preincubated enzyme mixture, 0.1 ml of freshly neutralized 0.3 M ascorbate, and 0.6 ml of distilled water. After mixing, the assay mixture (3.0 ml) was immediately placed in the sample chamber of the spectrophotometer, equilibrated at 30°C and the initial change in absorbance due to kynurenine formation was followed at 360 nm for at least 15 minutes. The extinction coefficient of kynurenine (absorbancy of 1 cm of 1.0 M solution at 360 nm) is 4530 (Knox, 1955a). Activities are expressed as units (μmoles of kynurenine formed per hour at 30°C) per gram of wet liver.

6. Protein determination

Protein was determined by the procedure of Lowry (1951). Protein samples were mixed with an equal volume of 1 N NaOH and digested at 60°C for three hours. One ml of the dilute protein solution was mixed with 5 ml of a copper reagent (2% Na_2CO_3, 0.01% $CuSO_4.5H_2O$ and 0.02% NaK tartrate) prepared fresh daily. Tubes were mixed and allowed to stand for 15 minutes. To each tube, 0.5 ml of 1 N phenol reagent (Folin and Ciocalteu's phenol reagent) was added and mixed thoroughly. After 10 minutes the absorbance at 660 nm was measured and compared to the standard.

The standards consisted of 1 ml samples of bovine serum albumin (20 to 200 μg/ml) in 0.5 N NaOH. These were treated in the same manner as the test proteins.

RESULTS AND DISCUSSIONS

PART I : Studies on the Stability of Pyridine Nucleotides

(A) Introduction

It is well known that reduced pyridine nucleotides may be destroyed by acid without damaging the oxidized forms (von Euler et al., 1936) and that, conversely, the oxidized pyridine nucleotides may be decomposed by alkali without the slightest loss of the reduced forms (Adler et al., 1936).

It is also known that pyridine nucleotides are rapidly destroyed in tissue homogenates by a heat-labile system (von Euler and Heiwinkel, 1937). The activity of this system can be inhibited by nicotinamide (Mann and Quastel, 1941).

The objectives of future studies are to establish the influence of niacin status on turnover rates of pyridine nucleotides and its effect on metabolically related enzymes . It is therefore important that the reliability of methods for the extraction and determination of pyridine nucleotide contents be examined. It is also particularly important to establish the influence of various conditions in tissue samples or homogenates prior to the denaturation of proteins.

The objective of the present study was to establish the effects on the stability of pyridine nucleotides of post-mortem changes, duration of homogenization time, and storage in the presence of reducing reagents.

(B) Experimental Procedures

EXPERIMENT 1 :

Mature male Japanese quail maintained on a chick starter diet (a complete diet) were sacrificed by decapitation. The liver was removed immediately and blotted free of blood. In general, livers were frozen within 3 minutes of decapitation in liquid nitrogen and stored at -70°C until analyzed.

A series of five trials were employed to investigate the stability of pyridine nucleotides in livers under a variety of conditions. In trial one, the recovery of pyridine nucleotides was determined when added to the liver during extraction and assay procedures. In trial two, the post-mortem change in pyridine nucleotides was examined by freezing liver tissues at 3 and 10 minute-intervals after decapitation. In this trial, liver samples were excised and kept in a small covered dish for 3 or 10 minutes at room temperature prior to freezing with liquid nitrogen. In trial three, the stability of pyridine nucleotides was tested using the fresh liver tissue versus the frozen liver tissue obtained at different time intervals. In trial four, the stability of pyridine nucleotides was tested following homogenization for 2 or 10 minutes with a Polytron homogenizer of liver tissues. In trial five, the stability of NADPH during storage was examined by the addition of reducing reagents to the homogenizing buffer. 2-Mercaptoethanol and reduced glutathione were added to the homogenizing buffer at the final concentrations

of 1mM respectively to determine if these compounds affected NADPH assay values. In this trial, the extracts were assayed at various time intervals throughout the course of experiment. The control sample assay did not contain a reducing reagent.

The methods for extraction and determination of pyridine nucleotides are described in General Experimental Procedures. Analysis of variance was performed according to Snedecor (1956) and treatment differences were subjected to the Students-Newman-Keuls multiple range test as outlined by Kirk (1968). In order to estimate the significance of differences between two means, the Student's t-test was employed (Hill, 1961).

(C) Results and Discussion

The results presented in Table 2 indicate a reasonably good recovery of NADH and NADPH when added to the extraction medium. The recovery of NAD and NADP however was not tested in this trial. Similar results were obtained in a second experiment in which known amounts of NAD and NADP were added to the assay system prior to being assayed. These results would suggest that there was a reasonably good recovery of pyridine nucleotides under both the extraction and assay procedures.

The data from Table 3 (trial 2) demonstrate that the concentrations of NAD, NADP, and NADPH were not greatly influenced by the time of freezing after the death of quail. NADH levels in all cases were too low to be detected.

Table 2. Recovery of Pyridine Nucleotides Added to Liver Tissue during Extraction and Assay Procedures (Experiment 1, Trial 1).*

Pyridine Nucleotides	RECOVERY OF PYRIDINE NUCLEOTIDES			
	Added during Extraction+		Added to Assay System	
	µmoles added	percentage recovered	µmoles added	percentage recovered
NAD	-	-	0.2	93.0±4.0
NADH	2.5	97.0±7.0	-	-
NADP	-	-	0.05	95.0±8.0
NADPH	4.0	90.0±5.0	-	-

*Values are mean ± S.E. of 2 observations.

+Pyridine nucleotides are added to the homogenizing buffer containing the liver tissues.

Table 3. Effect of Freezing Time on Stability of Coenzymes at Various Time Intervals after Sacrifice (Experiment 1, Trial 2).*

Time Between Decapitation and Freezing of Liver Tissue (min)	Coenzyme Levels (µmoles/ g liver)			
	NAD	NADP	NADH	NADPH
3	0.60±0.06	0.13±0.01	0	0.48±0.03
10	0.50±0.08	0.10±0.02	0	0.50±0.08
P	P>0.05	P>0.05	P>0.05	P>0.05

*Values are mean ± S.D. of 3 observations.

The results from Table 4 (trial 3) demonstrate there were no appreciable differences in coenzyme levels between fresh or frozen tissues (-70°C) or between frozen tissues when stored for various time intervals. In another study it was shown that NADPH levels tended to decline as the time of storage at -70°C was prolonged.

The results from Table 5 (trial 4) indicate that homogenization time (2 versus 10 minutes) tended to reduce the levels of NAD, NADP, and NADPH. These results would suggest that homogenization time is not highly critical but that it should not be excessively long. The mean non-significant ($p > 0.05$) decrease in NAD, NADP, and NADPH concentrations due to a five-fold increase in homogenization time was 20%, 33%, and 9.3%, respectively.

The results from Table 6 (trial 5) indicate that 2-mercaptoethanol was a more effective NADPH protecting reagent than reduced glutathione ($p < 0.01$). Nevertheless, reduced glutathione protected NADPH to a slight degree relative to the control which did not contain any reducing reagents ($p < 0.05$). However, there was a tendency for NADPH to decompose gradually over a period of 8 days at 4°C irrespective of the presence of reducing reagents in the extract.

Polakis and Bartley (1966) observed that cysteine, added to weak acid used in preparation for the yeast extracts, yieded lower NADP values, presumably by preventing NADPH oxidation. Neubert et al. (1964) reported that re-

Table 4. Stability of Coenzymes during Storage (Experiment 1, Trial 3).*

Pyridine Nucleotides	Coenzyme Levels (μmoles/g liver)			
	Fresh Tissue (3 min)	Frozen Tissue (30 hr)	Frozen Tissue (72 hr)	P
NAD	0.59±0.07	0.57±0.05	0.63±0.08	p>0.05
NADP	0.09±0.02	0.09±0.02	0.11±0.02	P>0.05
NADH	0	0	0	P>0.05
NADPH	0.56±0.05	0.53±0.05	0.52±0.05	P>0.05

*Values are mean ± S.D. of 2 observations.

Table 5. Effect of Homogenization Time on Stability of Coenzymes (Experiment 1, Trial 4).*

Homogenization Time (min)	Coenzyme Levels (μmoles/g liver)			
	NAD	NADP	NADH	NADPH
2.0	0.61±0.11	0.09±0.01	0	0.54±0.04
10.0	0.50±0.01	0.06±0.01	0	0.49±0.04
P	P>0.05	P>0.05	P>0.05	P>0.05

*Values are mean ± S.D. of 2 observations.

Table 6. Stability of NADPH in the Presence of Reducing Reagents (Experiment 1, Trial 5).*

Time Elapsed (day)	NADPH (μmoles/g liver)		
	Without Reducing Reagents	1 mM 2-Mercaptoethanol	1 mM GSH
0	0.59±0.04	0.77±0.04	0.66±0.04
1	0.48±0.04	0.67±0.04	0.57±0.04
2	0.36±0.02	0.62±0.04	0.44±0.02
3	0.29±0.01	0.55±0.04	0.37±0.02
4	0.28±0.01	0.51±0.04	0.32±0.02
5	0.22±0.01	0.42±0.02	0.23±0.01
8	0.09±0.01	0.27±0.01	0.14±0.01

*Values are mean ± S.D. of 2 observations.

Abbreviations: GSH, reduced glutathione.

duced glutathione was partially protective against the oxidation of NADPH when liver mitochondria were acidified. According to studies by Burch et al.(1967), all sulfhydryl compounds (ie. cysteine, dithiothreitol, 2-mercaptoethanol and reduced glutathione) were rather ineffective whether added to the acid (up to 8mM concentration) or to an alkaline mixture of hemoglobin and NADPH prior to acidification (final sulfhydryl concentration was up to 1.5 mM).

The pH stability was not studied as this has been well established in the literature. NAD is stable in dilute acids, but unstable in alkali whereas NADH is stable in dilute alkali, but unstable in acids (Schlenk, 1951). Although NADP is relatively stable in weak acids, it is rapidly destroyed in alkaline solution with the cleavage of nicotinamide (Kaplan et al.,1951). NADPH is relatively stable in alkaline conditions, but is destroyed on exposure to acid due to the opening of the pyridine ring (Kaplan, 1960).

Surprisingly in the current study no NADH was detected in the liver in any of the subsequent studies. Since there was a good recovery of NADH when added to the extraction medium it is assumed that the level of NADH in liver is too low to be determined. Although it is generally believed that the level of NADH in pigeon is very low in all organs relative to other pyridine nucleotides (Jedeikin and Weinhouse, 1955), it is rather intriguing to explain the phenomenon in which no NADH was detected in the current assay system.

Jandorf (1943) reported that the initial value of NAD per gram rat liver decreases by 10 and 19%, respectively within 2.5 and 15 minutes of killing and that it becomes stable after 20 minutes. It was also speculated that the decline of NAD during the first few minutes was probably due to a conversion of NAD to NADH as a result of anaerobiosis. The data from the current study, although not significantly different, follow the same trend.

In a very similar experiment to Jandorf (1943), Jedeickin and Weinhouse (1955) observed that there was a gradual decrease in NAD, amounting to 29% in 1 hour, 49% in 2 hours and 60% in 4 hours after standing at room temperature. A similar decrease in NADH was also reported. From these findings, they suggested that large variations in the amounts or proportions of the two forms of the pyridine nucleotide would not be expected during the 5 or 10 minutes ordinarily required for removal of tissues. More precise information with regard to relative proportions of reduced and oxidized forms of the coenzymes would require that tissues should be frozen within a few seconds of death. The freeze clamp technique of Wollenberger et al.(1960) could be used for this purpose. This technique was not utilized in the current study as the initial concern of the study was to determine total amounts of oxidized and reduced form of the two coenzymes and not proportions of the oxidized and reduced forms.

The results of the current study would suggest that under the conditions tested there is a slow progressive decrease in pyridine nucleotide concentration during the assay procedure. Factors that influence this would include the time after freezing, duration of the homogenization period and the level of reducing reagents in the extracting media.

PART II : Studies on the Induction of Niacin Deficiency by Sulfaguanidine and Amino Acid Imbalances

(A) Introduction

Many reports have been published regarding the induction of niacin deficiency by various means in several species. It has been postulated that an excess of leucine is a possible causative factor in the development of pellagra (Gopalan and Srikantia, 1960) and isoleucine counteracts the pellagragenic effect due to high leucine (Krishnaswamy and Gopalan, 1971) . It has also been well established that an excess of dietary leucine retarded the growth of rats and the addition of isoleucine overcame the growth depressing action of the excess leucine (Harper et al., 1955). Niacin was not a limiting nutrient in this latter experiment.

In contrast to the work carried out with humans, it has recently been demonstrated that an excess of leucine is not primarily responsible for the induction of niacin deficiency in rats or chicks (Nakagawa et al., 1977; Mason and Carpenter, 1978). It has also been postulated that microbial synthesis of niacin may be an important source of niacin as the bacteriostatic agent, sulfaguanidine, when fed to the young rat greatly reduces growth rate. It was assumed that intestinal bacteria were able to meet part of the animal requirements for niacin (Black et al., 1947).

The initial experiment outlined in this section involves mature quail rather than immature quail in order to

facilitate the subsequently planned protein turnover studies. The reason for this requirement is that protein turnover is most suitably measured in animals that are in a steady state condition (ie. non-growing state)(Arias et al., 1969). In the subsequent experiment, immature quail were employed in an attempt to accelerate the onset of niacin deficiency as it was not possible to develop this deficiency in mature quail. Biochemical parameters which were thought to be an index of niacin deficiency were also measured. In both experiments, a high level of leucine was used in order to accentuate niacin deficiency and isoleucine was added in an attempt to correct this disturbance. Sulfaguanidine was also used in the first experiment.

(B) Experimental Procedures

EXPERIMENT 2 : MATURE QUAIL

Prior to the initiation of this experiment , mature female Japanese quail, four months old, were fed ad libitum a mixed diet (chick starter plus niacin free diet) for 10 days. During this adaptation period the relative amount of the niacin free diet in a mixed diet was gradually increased.

After this period, 127 to 128 g quail were divided into eight groups of seven quail each. Each group was fed the basal diet (contained no added nicotinic acid) plus supplemental levels of the following compounds : diet 1, 0.007 % nicotinic acid; diet 2, no additives; diet 3, 0.5% sulfa-

guanidine; diet 4, 0.5% sulfaguanidine plus 3% leucine; diet 5, 3% leucine; diet 6, 3% leucine plus 1% isoleucine; diet 7, 6% leucine; and diet 8, 6% leucine plus 2% isoleucine. The quail were allowed free access to food and water. Body weights were determined every week and mortality was checked daily throughout the experiment. At the end of the experiment, all quails were sacrificed by decapitation and the livers were removed and stored at -70°C until analyzed.

Pyridine nucleotides were analyzed by methods described in General Experimental Procedures. Analysis of variance was conducted according to Snedecor (1956) and treatment differences were subjected to Students-Newman-Keuls multiple range test as outlined by Kirk (1968).

EXPERIMENT 3 : IMMATURE QUAIL

Newly hatched quail were fed a chick starter diet until they reached 3 weeks of age. They were then provided ad libitum a mixed diet consisting of chick starter and the niacin free diet for 1 week. During the adaptation period the relative amount of niacin free diet in the combined diet was gradually increased.

Quail weighing from 55 to 62 g were then randomly divided into six groups of 12 quail each. The six dietary treatments included the basal diet (niacin free diet) plus supplemental amounts of the following ingredients : diet 1, 0.007% nicotinic acid; diet 2, no additives; diet 3, 3% leucine ; diet 4, 3% leucine plus 1% isoleucine; diet 5, 6% leucine ;

and diet 6, 6% leucine plus 2% isoleucine. All quail had free access to food and water for 8 weeks and body weights were measured every week.

The quail were sacrificed after 8 weeks by decapitation and livers were promptly removed, frozen in liquid nitrogen and stored at -70°C until analyzed. Chemical and statistical analysis were the same as in Experiment 2 and General Experimental Procedures.

(C) Results and Discussion

The growth curves for mature female quail fed the eight different diets in experiment 2 are illustrated in Figure 9. The results demonstrated that after an initial rapid decline in weight during the first 2 weeks, average body weights of quail on the eight diets did not change appreciably during the remaining 5 week period. Quail on the control diet, however, did not lose as much weight initially or throughout the experiment as compared to those on the other diets ($p < 0.05$). The expected weight depressing effects of sulfaguanidine and leucine did not occur and the addition of isoleucine to the leucine supplemented diets had no effect.

A comparison of body weight changes, liver weights, and pyridine nucleotide levels of each group of quail also demonstrated no significant differences ($p > 0.05$) among the eight treatments (Table 7). Results from the 8 week experiment would suggest that it is not possible to induce niacin

Figure 9. Average Growth Curves for Mature Female Quail fed the Following Diets below (Experiment 2).

▽ : (1) NFD + 0.007% nicotinic acid (control)

○ : (2) NFD

⊡ : (3) NFD + 0.5% sulfaguanidine

⊙ : (4) NFD + 0.5% sulfaguanidine + 3% leucine

◺ : (5) NFD + 3% leucine

□ : (6) NFD + 3% leucine + 1% isoleucine

△ : (7) NFD + 6% leucine

✕ : (8) NFD + 6% leucine + 2% isoleucine

Each point represents mean of 5 observations. Standard errors were not shown for the clarity of curves.

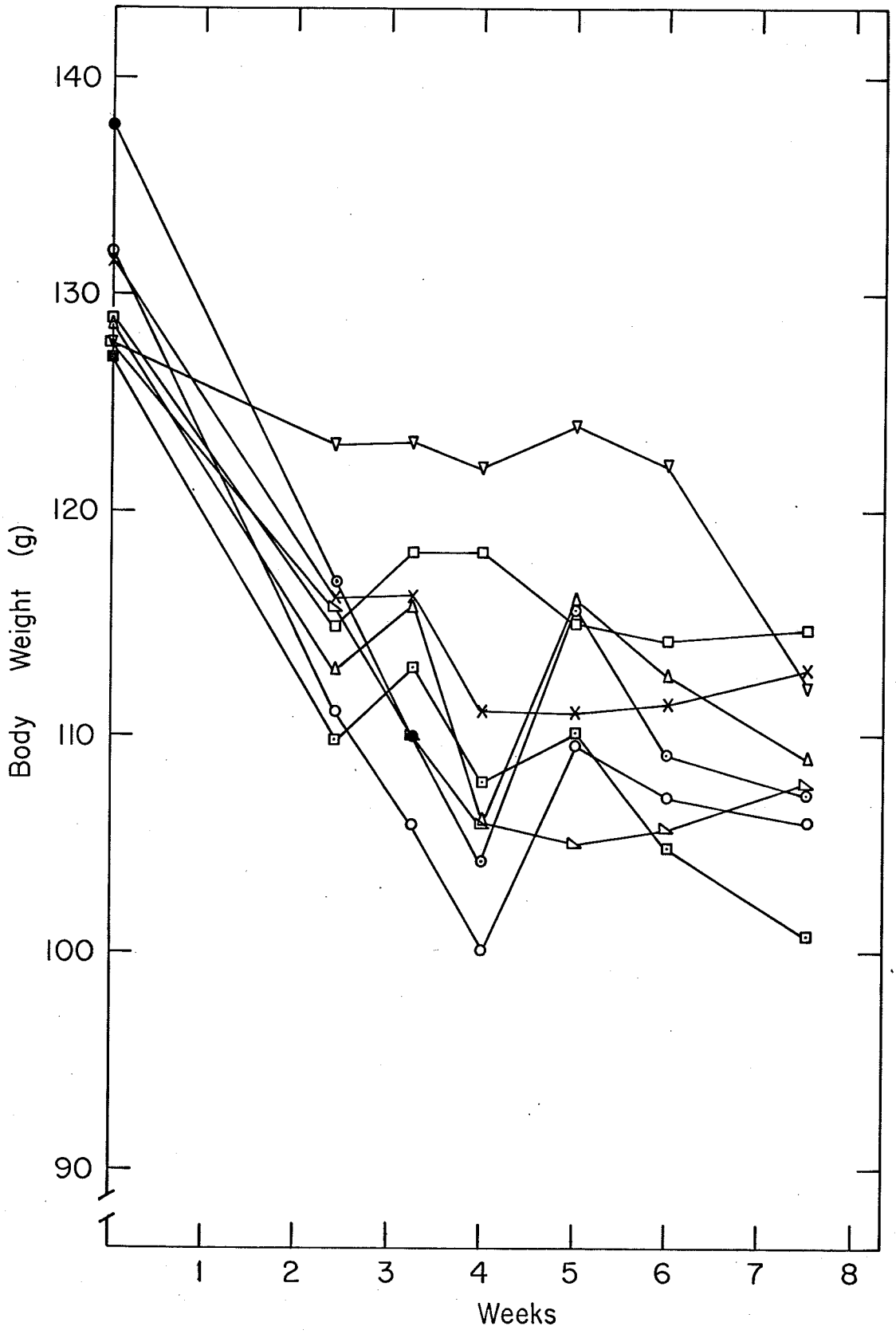

140
130
120
110
100
90
Body Weight (g)
1
2
3
4
5
6
7
8
Weeks

Table 7. Effects of Amino Acid Supplementation and an Antimicrobial Agent on Body Weight, Liver Weight, and Pyridine Coenzyme Levels of Mature Female Japanese Quail (Experiment 2).[1]

Diet	Initial BW	Final BW	BW Change	Liver Wt	No. of Mortality	N A D		N A D P H		N A D P	
	g	g	%	g		µmoles/g liver	µmoles/100 g BW[2]	µmoles/g liver	µmoles/100g BW	µmoles/g liver	µmoles/ 100g BW
1) NFD + 0.007% nicotinic acid	128±6.0	112±11	-12.5±1.7	3.37±0.61	0	0.61±0.13	1.84±0.22	0.50±0.06	1.50±0.30	0.091±0.01	0.27±0.03
2) NFD	132±2.0	106±4.0	-19.7±2.4	3.18±0.32	0	0.56±0.13	1.68±0.21	0.52±0.10	1.56±0.34	0.082±0.01	0.25±0.03
3) NFD + 0.5% Sulfaguanidine	127±8.0	100±5.0	-21.3±1.9	2.75±0.50	1	0.62±0.06	1.71±0.27	0.52±0.06	1.44±0.26	0.095±0.01	0.26±0.05
4) NFD + 0.5% sulfa-guanidine + 3% leu	138±8.0	107±7.0	-22.5±2.5	3.07±0.50	0	0.58±0.09	1.66±0.30	0.45±0.05	1.32±0.20	0.085±0.01	0.24±0.02
5) NFD + 3% leucine	128±5.0	108±4.0	-15.6±1.5	3.11±0.41	2	0.54±0.05	1.56±0.34	0.47±0.03	1.35±0.15	0.081±0.01	0.23±0.04
6) NFD + 3% leucine +1% isoleucine	129±6.0	115±2.0	-10.9±1.8	3.25±0.48	0	0.58±0.03	1.64±0.23	0.50±0.06	1.41±0.31	0.079±0.01	0.22±0.02
7) NFD + 6% leucine	129±4.0	109±3.0	-15.5±1.0	2.87±0.20	0	0.67±0.04	1.76±0.20	0.52±0.10	1.37±0.30	0.073±0.01	0.19±0.03
8) NFD + 6% leucine + 2% isoleucine	132±5.0	113±3.0	-14.4±1.4	2.84±0.23	0	0.59±0.04	1.48±0.21	0.48±0.04	1.22±0.20	0.098±0.01	0.25±0.04

[1] Values are means ± S.E of 5 observations each, values within column were not significantly different (P>0.05).

[2] µmoles/100 g BW is calculated by multiplying µmoles/g liver by multiplying the total liver weight by 100 and by dividing by body weight.

Abbreviations: NFD, niacin free diet; BW, body weight; Wt, weight.

deficiency symptoms in mature female Japanese quail under the condition used in this experiment and that the expected niacin deficiency was not enhanced in the presence of leucine or sulfaguanidine.

In experiment three, immature Japanese quail weighing from 55 to 62 g were used to study the effects of leucine and isoleucine imbalance on body weight gain, liver weight, levels of pyridine nucleotides and enzymes activity levels. The growth curves for quail fed the six diets demonstrated that there were considerable differences among the groups in rates of body weight gain (Figure 10). Quail fed the niacin free diet had a growth rate which was significantly lower ($p < 0.01$) than that of quail fed the nicotinic acid supplemented diet but were significantly higher ($p < 0.01$) than for those fed the other diets. In all cases, there was either no change or a decrease in body weight during the latter portion of the experiment when quail were fed the various nicotinic acid free diets. An overall summary of the data is presented in Table 8. There were significant differences in body weight gains between the control group fed 0.007% nicotinic acid and quail fed the niacin free diet ($p < 0.01$) and between the control group and the other groups ($p < 0.01$). Quail in the latter group also had significantly lower ($p < 0.01$) body weight gains than those fed the niacin free diet. This indicates that the inclusion of leucine as well as leucine plus isoleucine in the niacin free diet partially depressed body weight gain.

Figure 10. Average Growth Curves for Immature Quail Fed the Following Diets Below (Experiment 3).

▽ : (1) NFD + 0.007% nicotinic acid

○ : (2) NFD

▷ : (3) NFD + 3% leucine

□ : (4) NFD + 3% leucine + 1% isoleucine

△ : (5) NFD + 6% leucine

× : (6) NFD + 6% leucine + 2% isoleucine

Each point represents mean of 8 observations. Standard errors were not shown for the clarity of curves.

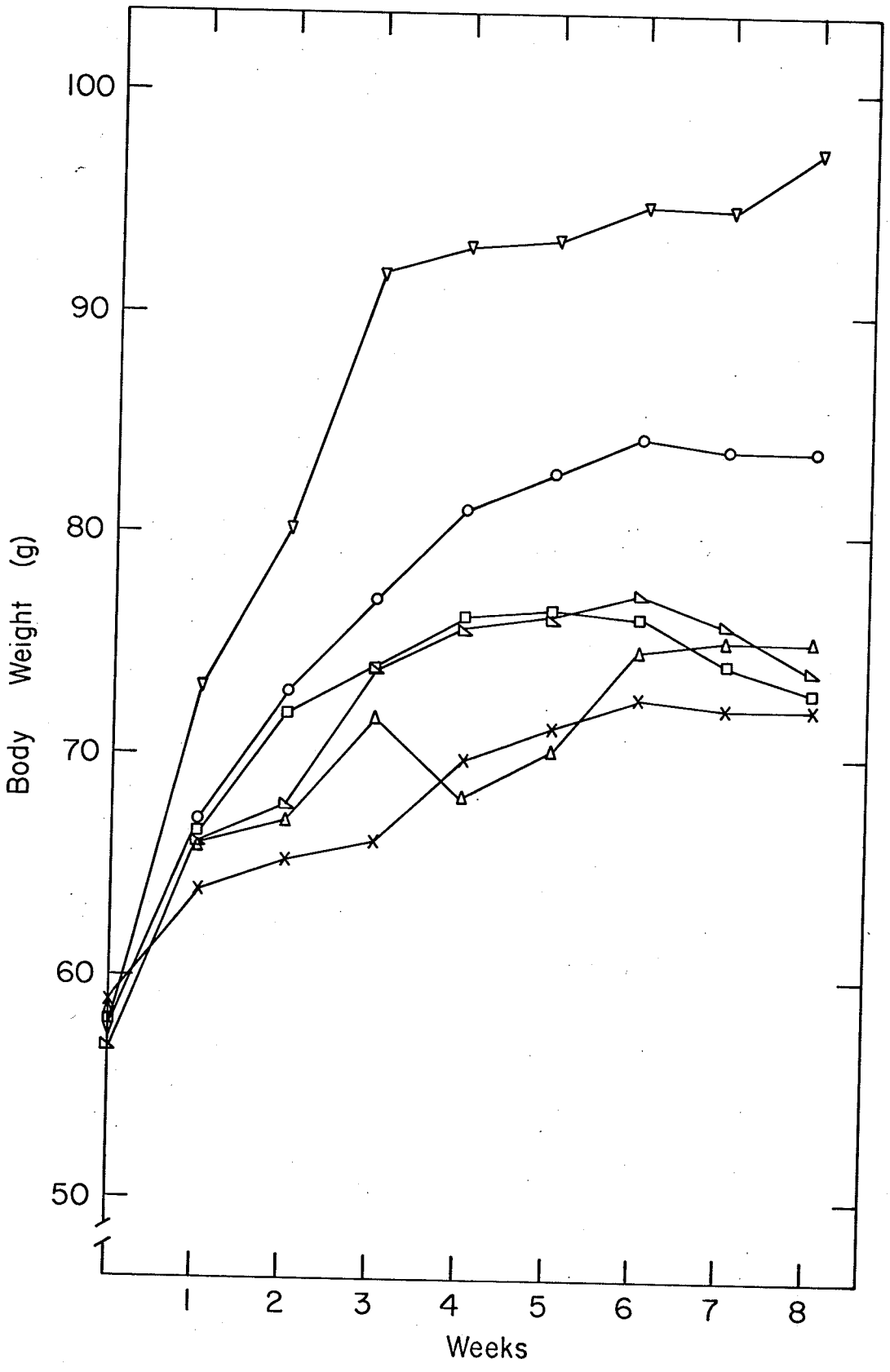

100
90
80
70
60
50
Body Weight (g)
1
2
3
4
5
6
7
8
Weeks

Table 8. Effects of Leucine and Leucine Plus Isoleucine on Body Weight Gain, Liver Weight, and Mortality in Immature Japanese Quail (Experiment 3).[1,2]

	Diet	Initial BW	Final BW	BW Gain	Liver WT	No. of Mortality
		g	g	g	g	
1)	NFD + 0.007% nicotinic acid	57.5±2.2	97.4±3.8^{A}	39.9±1.5^{A}	1.86±0.13	1
2)	NFD	57.4±1.0	84.0±4.0^{B}	26.6±1.5^{B}	1.76±0.12	2
3)	NFD + 3% leucine	56.8±2.5	73.7±4.4^{C}	16.9±1.4^{C}	1.61±0.09	3
4)	NFD + 3% leucine + 1% isoleucine	58.3±1.9	73.2±4.2^{C}	14.9±0.6^{C}	1.82±0.12	3
5)	NFD + 6% leucine	58.5±1.9	74.4±4.5^{C}	15.9±0.4^{C}	1.65±0.06	2
6)	NFD + 6% leucine + 2% isoleucine	59.0±1.7	72.1±4.5^{C}	13.1±0.8^{C}	1.93±0.25	3
		P>0.05			P>0.05	

[1]Values are means ± S.E. of 8 observations.

[2]Means not sharing a common superscript letter within a column are significantly different (P<0.01).

Abbreviations: NFD, niacin free diet; BW, body weight; wt, weight; no, number.

Some of quail that were fed the niacin free diet, the niacin free diet supplemented with leucine or the niacin free diet supplemented with leucine plus isoleucine exhibited poor feathering as compared to those from the control group. These symptoms are indicative of niacin deficiency (Scott et al., 1976).

In the case of total liver weights or pyridine nucleotide levels, no significant differences ($p > 0.05$) were observed among the six treatment groups. Similar non-significant differences ($p > 0.05$) were also obtained with the activity levels of NADP-dependent malic enzyme, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, or aldolase which does not require these coenzymes (Tables 9 and 10).

The results obtained in the current study with sulfaguanidine are different than those reported by Marshall et al. (1940) and Black et al. (1947). Marshall et al. postulated that sulfaguanidine could be an effective bacteriostatic agent for intestinal bacteria which were responsible for the synthesis of the vitamin B complex. Black et al. demonstrated that the growth rate of rats was reduced from the average 25 g per week to less than 10 g per week when 0.5% of this drug was added to regular synthetic diet. The failure to obtain similar results with mature quail may be attributed to the fact that their requirements for niacin may be lower than that of the rat or that some other factors influenced their requirements. These results nevertheless would suggest that niacin is very

Table 9. Effects of Leucine and Leucine Plus Isoleucine on Pyridine Coenzyme Levels in Liver Tissue of Immature Japanese Quail (Experiment 3).[1]

Diet	NAD		NADP		NADPH	
	µmoles/g liver	µmoles/100 g BW[2]	µmoles/g liver	µmoles/100 g BW	µmoles/g liver	µmoles/100 g BW
1) NFD + 0.007% nicotinic acid	0.69±0.06	1.32±0.18	0.17±0.04	0.32±0.07	0.56±0.04	1.07±0.14
2) NFD	0.58±0.06	1.22±0.18	0.14±0.01	0.29±0.04	0.53±0.02	1.11±0.09
3) NFD + 3% leucine	0.64±0.07	1.39±0.20	0.13±0.02	0.28±0.05	0.63±0.04	1.38±0.19
4) NFD + 3% leucine + 1% isoleucine	0.60±0.06	1.49±0.20	0.13±0.02	0.32±0.05	0.53±0.06	1.32±0.21
5) NFD + 6% leucine	0.73±0.07	1.57±0.25	0.20±0.06	0.45±0.09	0.59±0.03	1.27±0.13
6) NFD + 6% leucine + 2% isoleucine	0.50±0.10	1.31±0.30	0.13±0.02	0.35±0.03	0.45±0.08	1.20±0.21

[1] Values are mean ± S. E. of 8 observations. Means within columns are not significantly different ($P>0.05$).

[2] µmoles/100 g BW are calculated by multiplying µmoles/g liver by multiplying the total liver weight by 100 and by dividing by body weight.

Abbreviations: NFD, niacin free diet; BW, body weight.

Table 10. Effects of Leucine and Leucine Plus Isoleucine on Enzyme Activities in Liver Tissue of Immature Japanese Quail (Experiment 3).[1]

Diet	Malic Enzyme		Glyceraldehyde-3-phosphate Dehydrogenase		Aldolase	
	units/g liver	units/100 g BW[2]	units/g liver	units/100 g BW	units/g liver	units/100 g BW
1) NFD + 0.007% nicotinic acid	23.9±1.0	45.7±4.0	151.6±7.5	289.6±33.9	107.2±9.9	204.7±23.5
2) NFD	26.5±2.0	55.5±5.3	136.3±6.8	285.6±18.6	96.2±12.0	201.6±19.0
3) NFD + 3% leucine	26.3±3.0	57.5±8.7	135.2±7.8	295.2±18.8	89.4±7.9	195.4±27.0
4) NFD + 3% leucine + 1% isoleucine	35.0±8.5	86.0±24.0	142.6±4.2	354.5±27.0	77.8±1.6	193.4±22.0
5) NFD + 6% leucine	22.5±4.0	48.3±7.5	143.7±13.3	308.1±43.7	87.3±9.6	187.3±11.1
6) NFD + 6% leucine + 2% isoleucine	19.3±4.0	51.7±6.3	117.7±15.8	299.0±25.0	72.2±13.1	193.3±24.0

[1]Values are mean ± S. E. of 8 observations. Means within columns are not significantly different ($P>0.05$).

[2]Units/100 g BW are calculated by multiplying units/g liver by multiplying the total liver weight by 100 and by dividing by body weight.

Abbreviations: NFD, niacin free diet; BW, body weight.

effectively conserved in the niacin deficient quail or that niacin was being provided from some other sources. Additional experiments should be carried out to more effectively compare the effects of dietary supplementation of sulfaguanidine in both mature and immature niacin deficient quail.

In current study, the failure to obtain a growth depressing effect in mature quail when leucine and/or isoleucine was added to the niacin free diet would suggest that they did not affect the niacin status of the animal. With immature quail both leucine or leucine plus isoleucine produced significant depressing effects on growth as compared to quail fed the niacin free diet ($p < 0.01$). It is likely that part of this apparent effect in immature quail may be attributed to an amino acid imbalance (Harper et al., 1955) rather than to niacin deficiency. However, additional experiments would have to be carried out to distinguish between these two effects.

Several researchers have reported conflicting results with regard to the influence of leucine and isoleucine in niacin deficiency. Gopalan and Srikantia (1960) and Krishnaswamy and Gopalan (1971) have postulated that high leucine levels in diets were pellagragenic and that isoleucine counteracts this effect. In support of this hypothesis Raghuramulu et al.(1965b) reported that the synthesis of nicotinamide nucleotides from nicotinic acid by erythrocytes was decreased in normal individuals fed a high level of leucine. Furthermore, Rao et al. (1972) indicated that leucine results in increase of picolinate carboxylase, tryptophan pyrrolase, and

decrease of quinolinate phosphoribosyl transferase which are responsible for the interference in tryptophan metabolism.

In contrast to Gopalan's reports, Allen et al.(1971) were unable to induce niacin deficiency in chicks with leucine supplements. Furthermore, Nakagawa et al. (1977) also supported Allen et al.s' view that leucine is not a primary agent in causing a niacin deficiency in rats. Very recently Mason and Carpenter (1978) carried out a comparable experiment to Allen et al.s' in order to establish the niacin status of chicks using a high level of leucine. It was found that chicks fed low levels of niacin and tryptophan produced niacin deficiency symptoms which were not exacerbated by the addition of supplementary leucine (17.4 g/kg diet). They also demonstrated that the addition of supplementary leucine to diets that contained optimum levels of niacin and tryptophan depressed food consumption and weight gains of chicks; then chicks, however, did not show any niacin deficiency symptoms. In addition, Pearson and Song (1963) reported that in the case of a disturbance in tryptophan-niacin metabolism by amino acid imbalance the metabolites affected appeared to be influenced by the age of animals.

In conclusion, the results obtained in the current study would suggest that it is difficult if not impossible to induce niacin deficiency in mature quails even when the synthesis of niacin by bacteria is suppressed by the administration of sulfaguanidine or by the addition of leucine to the diet. The results obtained with immature quail demon-

strated that it is possible to induce niacin deficiency symptoms in these birds. A rather long period of time, however, was required to cause cessation of growth. These effects in both the mature and immature quail may be attributed to the fact that niacin is synthesized from either microbial sources or tryptophan derived from tissue proteins or the diet, and/or that niacin is very effectively conserved in the the animal. The overall results would suggest that a re-evaluation of niacin requirements be made in quail and other avian species , particularly with regard to mature birds. Niacin deficiency however did not affect any of the biochemical parameters including NAD and NADP that were measured in the liver of the immature bird. It is conceivable that niacin levels in the liver of the niacin deficient bird are maintained at a constant level at the expense of other tissues.

PART III : Effects of Niacin Deficiencies on Pyridine Nucleotide Levels and Enzyme Activities in Different Organs of Immature Quail

(A) Introduction

Briggs et al. (1943) demonstrated that chicks receiving purified rations low in nicotinic acid showed poor growth, a decreased food consumption, a marked lowering of the nicotinic acid and NAD content of breast muscle, poor feather development, and perosis or scaly dermatitis. Williams et al. (1950) also showed that NAD levels in the liver from immature rats fell to very low values when tryptophan and niacin were excluded from the diet. Feigelson et al. (1951) observed that nicotinic acid and tryptophan were equally effective as precursors of liver pyridine nucleotides in rats. Garcia-Bunuel et al. (1962) reported the response of pyridine nucleotides and pyridine nucleotide requiring dehydrogenases in the brain and liver of rats fed a low tryptophan, niacin free diet. The niacin deficient rat had reduced levels of brain pyridine nucleotides but the activities of glutamic dehydrogenase and isocitrate dehydrogenase were unchanged. A similar reduction of pyridine nucleotides was observed in the liver. Isocitrate dehydrogenase and α-glycerophosphate dehydrogenase activities were 25% and 50% less respectively in niacin deficient liver whereas glutamic dehydrogenase activity was unchanged. Katunuma et al. (1973) reported that a group-specific protease for NAD- or NADP- requiring enzymes was enhanced in niacin deficient

condition.

Although information, as indicated above, is available on some of the effects of niacin deficiency a considerable amount of additional research must be carried out to more clearly establish overall effects and sequence of events that occur during the development of niacin deficiency. The results of the previous experiments also indicated that it was not possible to induce niacin deficiency symptoms in mature quail and that these symptoms did not readily develop in immature quail that had reached 65% of mature weight.

The objectives of these studies were to develop procedures for the induction of niacin deficiency in the immature quail on a routine basis and to establish corresponding changes in pyridine nucleotide and enzyme activity levels of several tissues.

Based on the rather poor results obtained from the previous investigations with both mature (an average initial weight of 131 g) and adolescent quail (an average initial weight of 58 g), one day old quail (an average weight of 7.2 g) were used in an attempt to induce niacin deficiency in Experiment 4. In Experiment 5, young growing quail (average initial weight of 28 g) were maintained on the niacin free diet for 3 weeks to determine if there was an adaptation to niacin deficiency. In Experiment 6, five different diets were fed to young growing quail (average initial weight of 20 g) for 2 weeks. In this last experiment a group of pair-fed control birds were also included in the study in order to

determine if the metabolic differences observed between the niacin deficient and the control groups were due to either niacin deficiency or to the accompanying inanition. Under these conditions it should be possible to preclude the effects of inanition alone as contributing the observed differences.

These investigations were undertaken to establish the response of growth, pyridine nucleotide contents, and enzyme activity levels to graded levels of nicotinic acid and to a high level of tryptophan in diets and to determine to what extent pyridine nucleotide contents in tissues would reflect the degree of nicotinic acid status in animals. For the determination of enzyme activities, four NAD- or NADP- requiring enzymes (malic enzyme, lactic dehydrogenase, glutamic dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase); tryptophan pyrrolase, an enzyme involved in tryptophan metabolism, and non-NAD or non-NADP requiring enzymes (aldolase and fructose diphosphatase) were measured.

(B) Experimental Procedures

EXPERIMENT 4 :

One day old quail weighing approximately 7.2 g were fed the niacin free diet (NFD) for 8 days. The chicks were kept in electrically heated stainless steel batteries with wire floors and had free access to water and diet. Body weight of the 120 chicks were recorded daily and symptoms of niacin deficiency were checked throughout the experimental

period.

EXPERIMENT 5 :

All newly hatched quail were offered a mixture of niacin free diet and chick starter diet (Feedrite) until they reached approximately 27 g. This procedure enabled the quail to adapt to the synthetic diet. During this adaptation period, the relative proportion of niacin free diet was gradually increased. After this period, the quail were separated into two groups of from 10 to 20 quail each and were fed the following diets : (i) niacin free diet plus 0.007% nicotinic acid, and (ii) niacin free diet. Other conditions were the same as in the previous experiment (Experiment 4).

EXPERIMENT 6 :

Newly hatched quail were maintained as described in Experiment 5 on a mixed diet consisting of the niacin free diet and the chick starter diet until they reached approximately 19 g. Then they were wing banded, weighed and divided into five groups of from 15 to 25 quail each and were fed the following diets : (i) diet 1 (Group I : control), niacin free diet plus 0.007% nicotinic acid ; (ii) diet 2 (Group II), niacin free diet ; (iii) diet 3 (Group III), the same diet as diet 1 ; (iv) diet 4 (Group IV), niacin free diet plus 0.66% tryptophan ; and (v) diet 5 (Group V), niacin free diet plus 0.035% nicotinic acid. The level of tryptophan used in diet 4 was three times higher than that for requirement (Scott et

al., 1959) and the level of nicotinic acid used in diet 5 was five times higher than that in diet 1.

All diets were fed ad libitum except for Group III, which received the diet once or twice a day so that the average weight of the group was the same as that in Group II . Birds in the former group were fasted for approximately 18 hours per day. Body weight and mortalities were recorded daily and signs of niacin deficiency were checked throughout the experiment. At sacrifice, the brain, heart, liver, and breast muscle were promptly excised, plunged into liquid nitrogen and weighed. Levels of pyridine nucleotides and enzyme activities of various organs were then measured as outlined in General Experimental Procedures. Analysis of variance was conducted according to Snedecor (1956) and treatment differences were subjected to the Students-Newman-Keuls multiple range test as outlined by Kirk (1968).

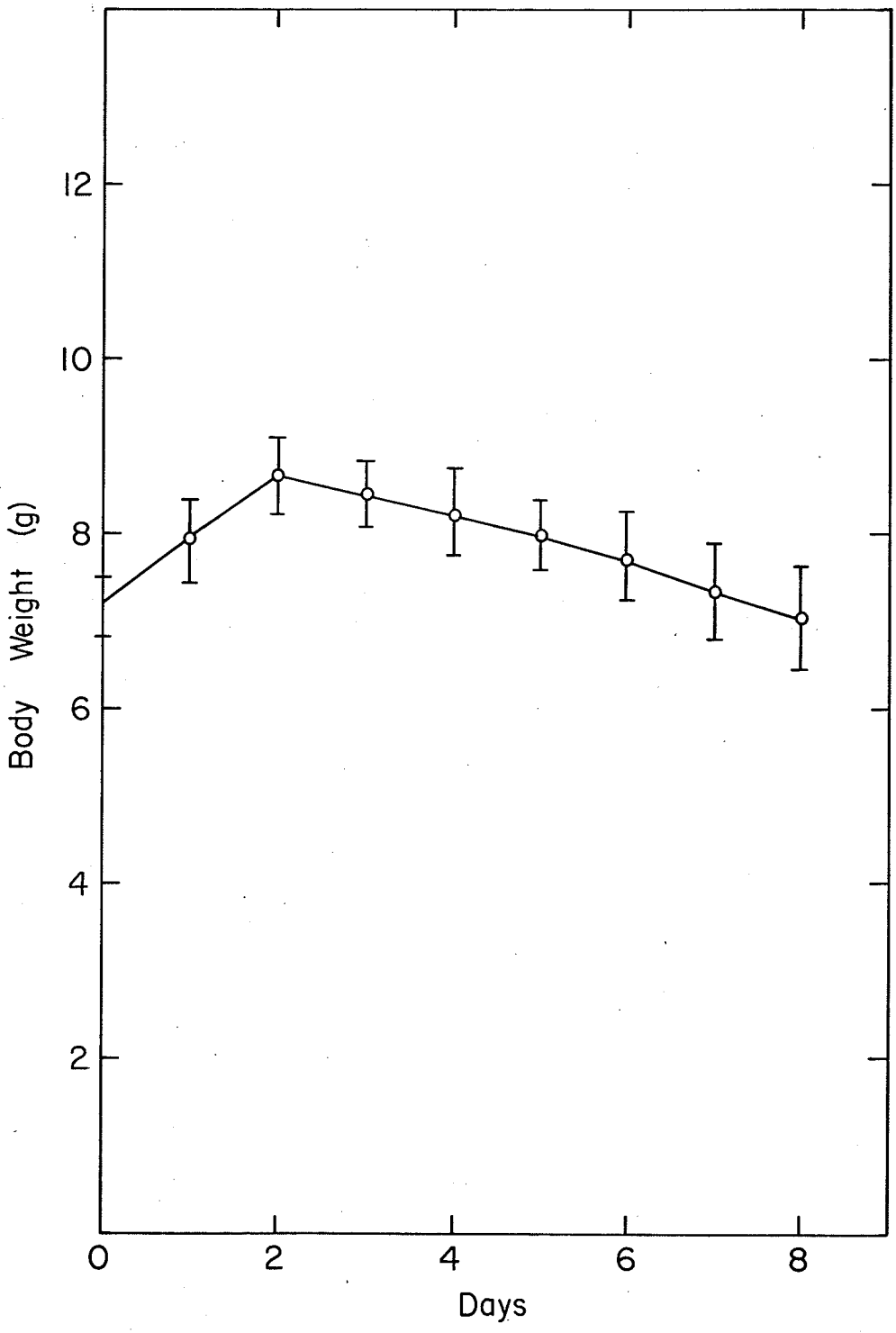
(C) Results and Discussion

EXPERIMENT 4 :

The growth curve of one day old quail fed the niacin free diet is illustrated in Figure 11. All chicks grew up to day 2 and then started losing body weight between day 2 and day 8, finally all died on day 9. The increase in growth during the initial two days may be ascribed to nicotinic acid retained in chicks after hatching rather than to the diet after which the chicks became nicotinic acid deficient. This

Figure 11. Growth Curve of One Day Old Quail Fed the Niacin Free Diet (Experiment 4).

The experiment was initiated with day old chicks. Each point represents the average body weight of 120 chicks. The bars represent standard error.


Body Weight (g)
12
10
8
6
4
2
0
2
4
6
8
Days

observation is in line with suggestions by Briggs and his coworkers (1942) that newly hatched chicks require nicotinic acid for optimal growth. In contrast, Dann and Handler (1941) reported that the hatched chicks do not require a dietary source of nicotinic acid for optimal growth as a result of synthesis of nicotinic acid during the incubation of egg. The results obtained in the current study re-emphasize the importance of inclusion of nicotinic acid in a diet for optimal growth of the immature chick.

EXPERIMENT 5 :

The growth curves for quail fed both the nicotinic acid supplemented (control) and the nicotinic acid free diets are presented in Figure 12. Quail fed the control diet grew at a steady rate whereas those fed the niacin free diet exhibited three stepwise growth patterns. Between days 0 and 7 very little or no gain in body weight was observed whereas between days 8 and 15 there was a slow rate of growth. In the final 16 to 21 day period there was a further increase in the rate of growth. These results strongly indicate that quail on the niacin free diet were not in a completely niacin deficient state as they tended to grow at a continually increasing rate. The source of niacin could be the diet, synthesis from tryptophan or by gut microflora.

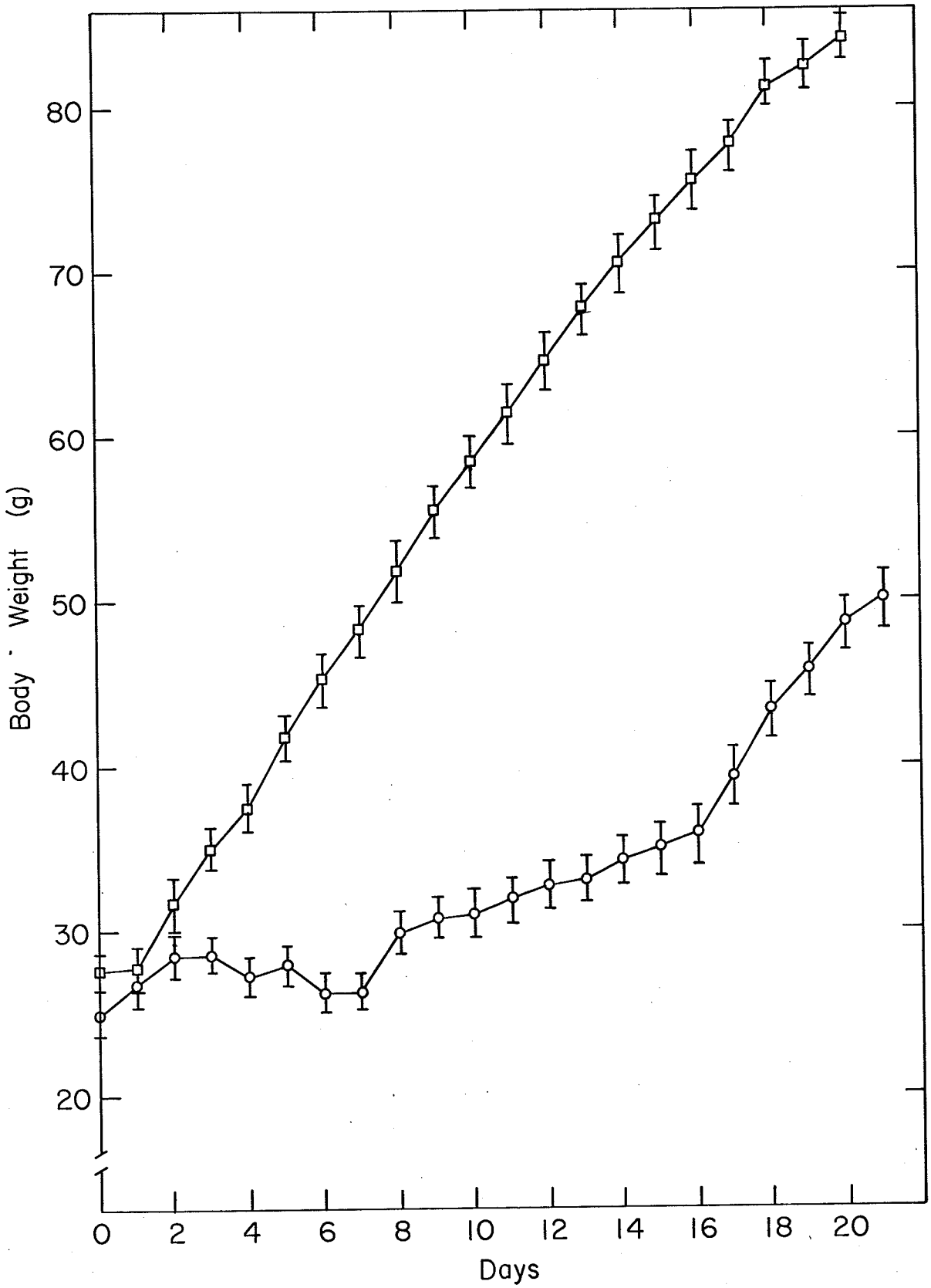
Chemical analysis of the diet and the fact that birds in the previous experiment died and those in the initial part

Figure 12. Growth Patterns of Quail Fed Nicotinic Acid Supplemented and Nicotinic Acid Free Diets (Experiment 5).

□: Group I (control) quail were fed ad libitum the NFD supplemented with 0.007% nicotinic acid

○: Group II quail were fed the NFD ad libitum

Quail were placed on test at 15 days of age. Each point represents the average body weight of 10 to 20 quail. The bars represent stand-error.


Body Weight (g)
80
70
60
50
40
30
20
0
2
4
6
8
10
12
14
16
18
20
Days

of the current experiment did not gain body weight would indicate that the diet was deficient in niacin. Although the level of tryptophan in the diet was only sufficient to meet amino acid requirements of birds, it is conceivable that under niacin deficiency conditions a portion of the tryptophan was diverted to the more essential production of niacin. The body reserves of niacin under these conditions would tend to be retained as previous results would suggest that the turnover of niacin was very low. Under these circumstances the birds would be able to grow at an ever increasing rate. The importance of gut microflora relative to tryptophan as a source of niacin can not be established from the results of the current experiment. Overall these results would suggest, nevertheless, that the duration of the feeding period and the selection of quail of proper age or size are crucial factors in the induction of niacin deficiency in quail. For example, in the initial experiment (Experiment 4) day old chicks were placed on a niacin free diet and they all lost weight and died within 9 days. In this last experiment, 15 day old quail (average body weight of 27 g) were placed on the same niacin free diet and they grew. In the subsequent experiment, birds were placed on the niacin free diet at a slightly younger age than in Experiment 5. The objective was to develop niacin deficiency symptoms without causing a change in body weight.

EXPERIMENT 6 :

The growth patterns of quail in response to various diets are depicted in Figure 13. The growth curves of groups IV and V were omitted as they were almost identical to that for Group I (control). It can be seen that quail receiving the niacin free diet (Group II), which had a very low level of nicotinic acid and an optimum level of tryptophan did not grow between days 3 and 15. The pattern of growth of these quail was very similar to the food restricted quail (Group III). In contrast, quail fed the nicotinic acid or the tryptophan containing diets (GroupI, IV, and V) grew at a steady rate and after 15 days they were two-fold heavier than the other two groups. Furthermore, the difference in growth rate was apparent within five days after the initiation of the experiment.

In addition to the typical growth depression, the symptoms of niacin deficiency seen in quail receiving the niacin free diet were a reduction in food consumption and certain outward manifestations such as slow feather development and blindness. It was also noted that niacin deficient quail had decreased voluntary activity and tended to huddle together in a corner of the cage relative to other groups. In a severe niacin deficiency state, the areas around eyes became darkened and very often both eyes were closed and covered with a crusty scale and exogenous secretions. To the best of our knowledge this peculiar phenomenon was never reported in the literature. Moreover, most of skull bones in the niacin deficient quail were very brittle in comparison to the other four groups (I, III, IV, and V). The pectoral

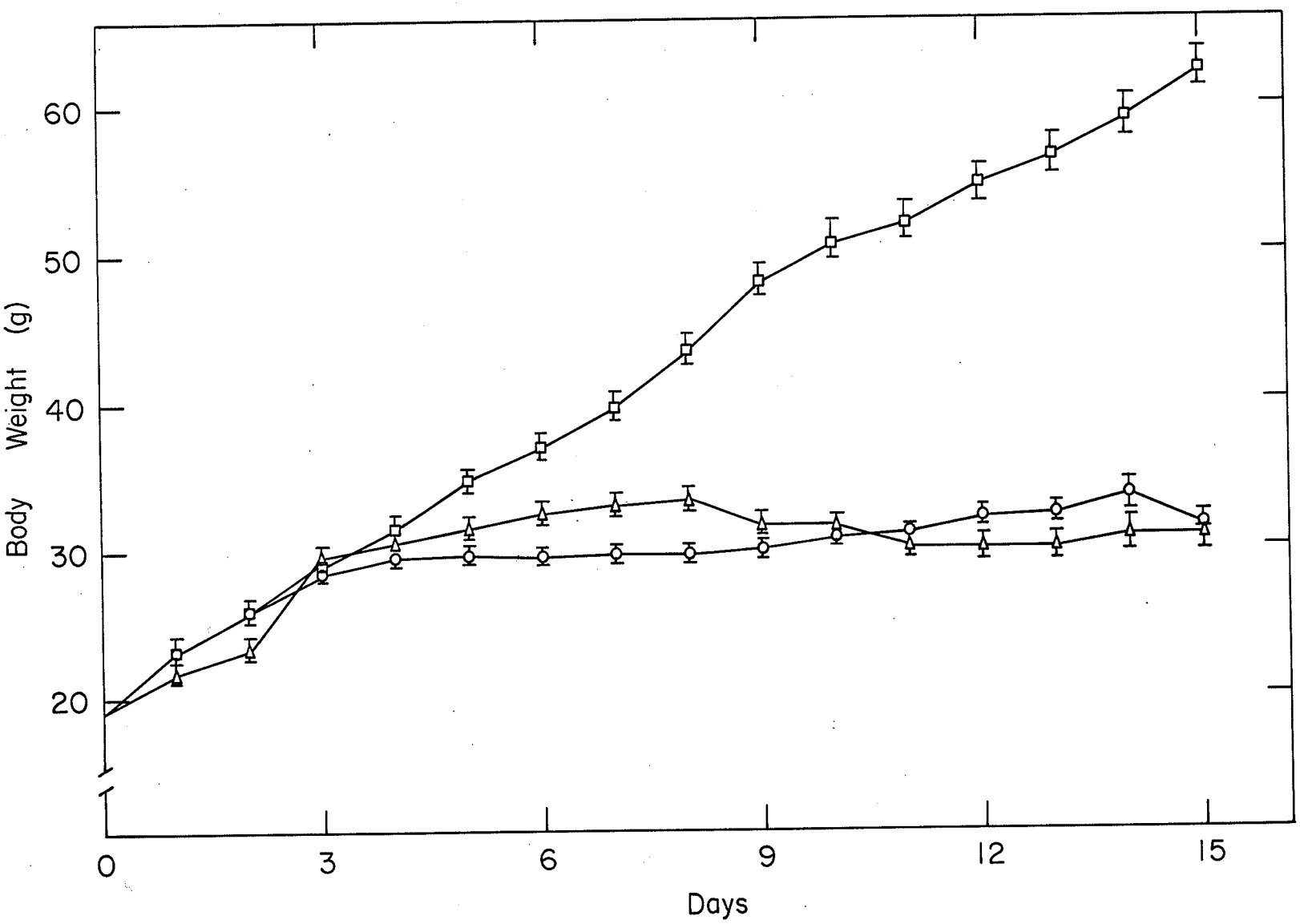
Figure 13. Growth Patterns of Quail Fed Various Diets (Experiment 6).

□ : Group I (control) quail were fed ad libitum the NFD plus 0.007% nicotinic acid

○ : Group II quail were fed the NFD ad libitum

△ : Group III quail were fed the same diet as group I except that the feed intake was restricted to that of Group II

Quail were placed on a test at 8 days of age. Each point represents the average body weight of 15 to 25 quail. The bars represent standard error.


Body Weight (g)
60
50
40
30
20
0
3
6
9
12
15
Days

muscle in the niacin deficient birds also appeared to be highly atrophied relative to both the control (Group I) and the two other groups (IV and V). This observation would suggest that muscle tissue are particularly sensitive to niacin deficiency.

Some of observations made in the current study are in accord with the results obtained in an earlier report by Briggs et al. (1942). They demonstrated that chicks receiving purified rations low in nicotinic acid grew poorly and developed a typical chick blacktongue. This latter condition was not observed in the current study. The results from the current study also showed that the diet which contained a higher concentration of nicotinic acid (0.035%) was no more effective than the 0.007% nicotinic acid containing diet in stimulating the growth of quail. Similar weight gains to those in Group I (control) were also obtained when the level of dietary tryptophan was increased three-fold. This would suggest that nicotinic acid requirements were completely spared by dietary tryptophan and that the level of tryptophan in the control diet was not limiting. Fisher et al. (1954) also reported that tryptophan completely spared nicotinic acid in the chick but that the reverse was not true. In contrast, Childs et al. (1952) reported that tryptophan did not totally spare nicotinic acid in young chickens.

The effects of five different dietary treatments on weight gain, liver, heart, and brain weights, mortality, and

protein concentrations of liver and breast muscle are summarized in Table 11. No mortality occurred within three of the groups (I, IV, and V) whereas during the time course of the experiment four quail died in both the niacin deficient and food-restricted groups respectively. Mean body weight gains of the food-restricted and the nicotinic acid deficient groups (II and III) were significantly ($p < 0.01$) less than those in the other groups (I, IV, and V). Similarly, average liver and heart weights of food-restricted and nicotinic acid deficient groups were significantly ($p < 0.05$) lower than those of groups I, IV, and V. The patterns of weight change for liver and heart were similar but less dramatic than that reported above. The differences in weights of liver, heart or body weight among the three groups (I, IV, and V), however, were not significant ($p > 0.05$). Brain weights in contrast were not affected by dietary treatments. It would appear however that the change in brain weight and to a lesser degree heart weight was not proportional to that of body weights. This is illustrated by the observations that the brain mass accounted for 1.7% of the total body weights in groups II and III but only 0.9% in the other groups. The tissue concentrations of protein in all cases were also similar as there were also no significant differences ($p > 0.05$) in protein concentrations of liver and breast muscle among the five groups.

Table12 , 13, and 14 show the content of pyridine nucleotides of liver, brain, heart, and breast muscle. There

Table 11. Effects of 5 Different Dietary Treatments on Body Weight, Weight Gain, Liver Weight, Heart Weight, Brain Weight, Mortality and Protein Content of Liver and Breast Muscle in Japanese Quail (Experiment 6).[1,2]

Group/Diet	Initial BW	Final BW	Wt Gain	Liver Wt	Heart Wt	Brain Wt	No. of Mortality	Protein level	
								Liver	Breast muscle
	g	g	g	g	g	g		g protein/g tissue Wt	
I NFD + 0.007% nicotinic acid (ad libitum)	19.2±0.9^{a}	62.3±1.2^{A}	43.1±1.0^{A}	1.91±0.06^{a}	0.53±0.04^{a}	0.60±0.01^{a}	0	0.29±0.01^{a}	0.24±0.01^{a}
II N F D (ad libitum)	19.5±0.7^{a}	31.4±1.7^{B}	11.9±1.5^{B}	1.05±0.08^{b}	0.39±0.02^{b}	0.52±0.01^{a}	4	0.28±0.01^{a}	0.23±0.01^{a}
III NFD + 0.007% nicotinic acid (restricted)	19.3±0.9^{a}	30.9±1.5^{B}	11.6±1.3^{B}	0.77±0.05^{b}	0.28±0.02^{b}	0.55±0.01^{a}	4	0.28±0.01^{a}	0.22±0.01^{a}
IV NFD + 0.66% tryptophan (ad libitum)	19.2±0.8^{a}	63.5±1.6^{A}	44.3±1.2^{A}	1.95±0.07^{a}	0.52±0.04^{a}	0.59±0.02^{a}	0	0.29±0.01^{a}	0.25±0.01^{a}
V NFD + 0.035% nicotinic acid (ad libitum)	19.2±0.9^{a}	62.2±1.7^{A}	43.0±1.2^{A}	1.75±0.05^{a}	0.47±0.04^{a}	0.56±0.01^{a}	0	0.28±0.01^{a}	0.24±0.01^{a}

[1]Values are mean ± S.E of 15-25 quail.

[2]Means not sharing a common superscript letter within a column are significantly different ($P<0.05$ or $P<0.01$).

Abbreviations: NFD, niacin free diet; BW, body weight; Wt, Weight.

Table 12. Effects of 5 Different Dietary Treatments on Levels of NAD, NADP, and NADPH in the Liver of Japanese Quail (Experiment 6), [1,2]

Group/Diet		NAD			NADP		
		µmoles/g liver	µmoles/g liver protein	µmoles/100 g BW[3]	µmoles/g liver	µmoles/g liver protein	µmoles/100 g BW
I	NFD + 0.007% nicotinic acid (ad libitum)	0.63±0.12[a]	2.7±0.45[a]	1.93±0.40[a]	0.21±0.01[a]	0.74±0.04[a]	0.66±0.05[a]
II	NFD (ad libitum)	0.63±0.10[a]	2.25±0.40[a]	2.11±0.40[a]	0.22±0.01[a]	0.78±0.03[a]	0.72±0.08[a]
III	NFD + 0.007% nicotinic acid (restricted)	0.72±0.11[a]	2.57±0.43[a]	1.79±0.30[a]	0.19±0.02[a]	0.68±0.07[a]	0.47±0.05[a]
IV	NFD + 0.66% tryptophan (ad libitum)	0.68±0.11[a]	2.34±0.42[a]	2.09±0.30[a]	0.20±0.02[a]	0.69±0.09[a]	0.63±0.07[a]
V	NFD + 0.035% nicotinic acid (ad libitum)	0.69±0.09[a]	2.46±0.40[a]	1.94±0.20[a]	0.22±0.02[a]	0.81±0.02[a]	0.63±0.06[a]

[1]Values are mean ± S.E for triplicate observations. Each triplicate consisted of liver samples pooled from 4 birds.

[2]Means not sharing a common superscript letter within a column are significantly different ($P<0.01$ or $P<0.05$).

[3]µmoles/100 g BW is calculated by multiplying µmoles/g liver by multiplying the total liver weight by 100 and by dividing by body weight.

Abbreviations: NFD, niacin free diet; BW, body weight.

Table 12. Continued.

Group	N A D P H			$\frac{NADP^*}{NADPH}$
	µmoles/g liver	µmoles/g liver protein	µmoles/100 g BW	
I	0.46±0.06[a]	1.59±0.26[a]	1.41±0.19[a]	0.46±0.03[a]
II	0.39±0.03[a]	1.39±0.12[a]	1.30±0.07[a]	0.56±0.04[a]
III	0.35±0.03[a]	1.25±0.10[a]	0.87±0.07[a]	0.54±0.03[a]
IV	0.38±0.04[a]	1.31±0.17[a]	1.17±0.19[a]	0.53±0.03[a]
V	0.45±0.05[a]	1.61±0.21[a]	1.27±0.15[a]	0.49±0.04[a]

* Nucleotide levels are expressed as µmoles per g liver.

were no significant differences ($p > 0.05$) irrespective of how the data were expressed (umoles per g liver weight, umoles per g liver protein, or umoles per 100 g body weight) in the content of liver NAD, NADP, and NADPH among the five groups. Also no significant change was observed in the ratio of NADP to NADPH among the five treatment groups. The results obtained in the current study are in agreement with the reports by Anderson et al. (1944) who demonstrated that no such definite correlation exists between the level of nicotinic acid in a diet and that of NAD in the liver of the rat. Morrison et al. (1960) also reported that nicotinic acid deficiency did not lead to a lowering of pyridine nucleotide concentrations of rat liver. It was also of interest to note that starvation for 48 hours (Pande et al., 1965) or starvation and re-feeding (Kayne et al., 1963) had no specific effect on pyridine nucleotide concentrations of rat liver.

In contrast with these observations, Glock and McLean (1955) reported that fasting for 48 hours in rats caused a marked reduction in total NAD (NAD + NADH). Similarly, Holzer et al. (1958) also found a reduction of NAD in livers of rats fasted for 24 hours. In addition, it was also demonstrated that rats receiving low amounts of nicotinic acid and tryptophan had subnormal levels of pyridine nucleotides in liver (Williams et al., 1950; Burch et al., 1955; Greengard et al., 1968).

NAD levels in the brain and heart of all five groups are summarized in Table 13. There were no significant differ-

Table 13. Effects of 5 Different Dietary Treatments on Levels of NAD in Brain and Heart Tissues of Japanese Quail (Experiment 6).[1,2]

Group/Diet		N A D			
		µmoles/g brain	µmoles/100 g BW[3]	µmoles/g heart	µmoles/100 g BW
I	NFD + 0.007% nicotinic acid (ad libitum)	0.38±0.04[a]	0.37±0.05[b]	0.49±0.06[a]	0.42±0.04[a]
II	NFD (ad libitum)	0.30±0.02[a]	0.50±0.04[a]	0.44±0.04[a]	0.55±0.09[a]
III	NFD + 0.007% nicotinic acid (restricted)	0.32±0.04[a]	0.57±0.08[a]	0.47±0.05[a]	0.43±0.03[a]
IV	NFD + 0.66% tryptophan (ad libitum)	0.32±0.03[a]	0.30±0.04[b]	0.49±0.08[a]	0.40±0.03[a]
V	NFD + 0.035% nicotinic acid (ad libitum)	0.33±0.04[a]	0.30±0.03[b]	0.55±0.10[a]	0.42±0.05[a]

[1]Values are mean ± S.E. for triplicate observations. Each triplicate consisted of heart or brain samples pooled from 4 birds.

[2]Means not sharing a common superscript letter within a column are significantly different ($P < 0.05$).

[3]µmoles/100 g BW is calculated by multiplying µmoles/g brain by multiplying the total brain weight by 100 and by dividing by body weight.

Abbreviations: NFD, niacin free diet; BW, body weight.

ences among the five groups studied in the levels of heart NAD irrespective of how the data are expressed (umoles per g heart or umoles per 100 g body weight). Similarly, no significant differences were detected in the level of brain NAD when expressed in umoles per g brain. However, when the level of brain NAD was expressed in umoles per 100 g body weight, the levels of brain NAD of both groups II and III were significantly ($p < 0.05$) higher than those of the remaining three other groups (I, IV, and V). This increase was attributed to the smaller body weight relative to brain size. It can also be seen that the inclusion of both nicotinic acid and tryptophan in excessive amount in the diet did not affect the levels of NAD in brain. Similar studies by Williams et al. (1950) and Brown (1964) reported that deficiencies of nicotinic acid and tryptophan in the diet had little or no effects on the levels of NAD in rat brain. In contrast, Garcia-Bunuel et al. (1962) presented data indicating that there was significant fall in NAD ($p < 0.001$), NADH ($p < 0.002$), and NADP ($p < 0.01$) levels in the brain of nicotinic acid deficient rats compared with controls.

In the current study, the levels of NAD and NADPH, but not NADP in the breast muscle of Japanese quail were markedly affected by dietary nicotinic acid levels (Table 14). NAD levels of quail fed the niacin free diet were either 66 or 63% lower ($p < 0.01$) than the other groups when compared on umoles per g breast muscle or umoles per g breast muscle protein basis. However, there were no significant differences

Table 14. Effects of 5 Different Dietary Treatments on Levels of NAD, NADP, and NADPH in Breast Muscle of Japanese Quail (Experiment 6).[1,2]

Group/Diet	NAD		NADPH		NADP	
	µmoles/g muscle	µmoles/g muscle protein	µmoles/g muscle	µmoles/g muscle protein	µmoles/g muscle	µmoles/g muscle protein
I NFD + 0.007% nicotinic acid (ad libitum)	0.96±0.03^{A}	4.05±0.29^{A}	0.122±0.009^{AB}	0.51±0.02^{ab}	0.025±0.003^{a}	0.11±0.01^{a}
II NFD (ad libitum)	0.35±0.09^{B}	1.61±0.50^{B}	0.017±0.009^{D}	0.08±0.05^{d}	0.024±0.002^{a}	0.11±0.01^{a}
III NFD + 0.007% nicotinic acid (restricted)	1.06±0.03^{A}	4.95±0.40^{A}	0.061±0.006^{C}	0.28±0.02^{c}	0.020±0.005^{a}	0.09±0.03^{a}
IV NFD + 0.66% tryptophan (ad libitum)	0.98±0.05^{A}	3.95±0.34^{A}	0.092±0.016^{BC}	0.37±0.07^{bc}	0.023±0.005^{a}	0.10±0.02^{a}
V NFD + 0.035% nicotinic acid (ad libitum)	1.09±0.03^{A}	4.65±0.25^{A}	0.145±0.008^{A}	0.62±0.06^{a}	0.020±0.008^{a}	0.09±0.04^{a}

[1]Values are mean ± S.E for triplicate observations. Each triplicate consisted of breast muscle samples pooled from 4 birds.

[2]Means not sharing a common superscript letter within a column are significantly different ($P<0.05$ or $P<0.01$).

Abbreviations: NFD, niacin free diet.

($p > 0.05$) in the NAD level among any of the other groups. The pattern of response in NADPH levels was similar to that for NAD except that there were also differences ($p < 0.01$) among several of the treatment groups. The average percent changes in NADPH levels per g breast muscle were as follows: 100% for Group I, 14% for Group II, 50% for Group III, 76% for Group IV, and 119% for Group V, respectively. Expressing values in umoles per g muscle protein yeild similar results.

These results would indicate that among the pyridine nucleotides assayed in the breast muscle NADPH appears to be the one most severely affected by a dietary deficiency of nicotinic acid. It was also of interest to note that the level of NADPH in the breast muscle of the the food-restricted group (III) was only half of that in the control group (I) and that NADP levels were not affected by any of the dietary treatments ($p > 0.05$). These results would suggest that breast muscle is the first tissue to exhibit a reduction in pyridine nucleotides in the niacin deficient birds and that the concentrations of either NAD or particularly NADPH are markedly affected. It is recommended,because of ease of assay, and the fact that the level of NAD does not decrease in the food-restricted bird that breast muscle NAD levels be used as an index of the niacin status of the bird. It may be hypothesized that a change in NADPH levels may be expected in the starved bird as this pyridine nucleotide is primarily associated with biosynthetic reactions (Atkinson, 1977) and that since the level of synthetic activity is low in the muscle tissue of

the starved animal there is a reduced need to have its level maintained. The results obtained in the current study are partially in agreement with the observations of Axelord et al. (1939) that nicotinic acid deficiencies in dogs and pigs resulted in a lower NAD content of both liver and muscle tissues. In support of these findings, Briggs et al. (1943) reported that chicks receiving purified diets free of nicotinic acid showed marked decreases of nicotinic acid and coenzyme I (NAD) contents in breast muscle. They did not, however, report NAD levels for other tissues including liver.

The activities of malic enzyme, lactic dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase in both liver and breast muscle tissues of all five groups were determined in the absence of their respective cofactors in the assay mixture. No enzymatic activities were detected in the absence of optimal concentrations of cofactors. It would appear that most of these NAD- and NADP- requiring enzymes may exist in the form of the apoenzymes, that they contain only small but undetectable amount of these coenzymes, or that the predominant species of bound coenzymes would be the opposite (oxidized form) to that used in the assay procedure. These results, nevertheless, appear to be different from that obtained with the pyridoxal phosphate requiring enzymes. Hunter and Harper (1976) reported that ornithine aminotransferase was predominantly in holoenzyme form in both pyridoxal phosphate deficient and control rats, whereas tyrosine aminotransferase was predominantly in apoenzyme form in both groups. However, the

proportion of serine dehydratase in apoenzyme form was twice as great as in the pyridoxal phosphate deficient as in the control liver of rats. These results would suggest that pyridoxal phosphate deficiency affects different enzymes in different ways, rather than generally causing the same effect and that different enzymes obviously have varying proportions of holo- and apo-enzyme forms which are independent of the tissue concentration of coenzyme.

The activity levels of liver enzymes in response to five different dietary treatments are summarized in Table 15. The activity of liver tryptophan pyrrolase of the food-restricted group (III) was approximately two-fold higher ($p < 0.01$) than that in the groups I, II, IV, and V whether expressed as units/hour/g liver or units/hour/g liver protein or units/hour/100 g body weight. However, there were no significant differences ($p > 0.05$) in enzyme activities among the four groups (I, II, IV, and V). The results of these studies would indicate that tryptophan pyrrolase activity was not affected by nicotinic acid status nor by dietary tryptophan levels but was affected by the type of feeding. This difference could be attributed to the fact that the food-restricted quail were fed once or twice per day and that they consummed all food within a few hours whereas all other groups consummed food throughout the day. This observation is consistent with that reported by Satyanarayana and Rao (1977) that liver tryptophan pyrrolase activity of rats increased as diet restriction was increased or by Wu and Rosenthal (1966) that the activity of

Table 15. Effects of 5 Different Diets on Tryptophan Pyrrolase, Fructose diphosphatase, Aldolase, Malic Enzyme, Glutamic Dehydrogenase and Lactic Dehydrogenase Activities of Liver Tissue in Japanese Quail (Experiment 6).[1,2]

Group/Diet		Tryptophan Pyrrolase			Fructose diphosphatase		
		units/hr/g liver	units/hr/g liver protein	units/hr/100 g BW	units/g liver	units/g liver protein	units/100 g BW[3]
I	NFD + 0.007% nicotinic acid (ad libitum)	1.49±0.16^{B}	5.16±0.58^{B}	4.57±0.42^{B}	9.6±0.3^{B}	33.4±1.7^{ab}	29.4±0.4^{ab}
II	N F D (ad libitum)	1.62±0.19^{B}	5.91±0.59^{B}	5.41±0.54^{B}	9.8±0.3^{B}	35.9±2.9^{ab}	32.1±1.5^{a}
III	NFD + 0.007% nicotinic acid (restricted)	3.15±0.08^{A}	11.36±0.47^{A}	7.85±0.50^{A}	10.9±0.2^{A}	39.3±1.5^{a}	27.1±0.7^{b}
IV	NFD + 0.66% tryptophan (ad libitum)	1.48±0.13^{B}	5.02±0.35^{B}	4.55±0.35^{B}	8.8±0.2^{B}	30.1±1.6^{b}	27.1±0.7^{b}
V	NFD + 0.035% nicotinic acid (ad libitum)	1.52±0.18^{B}	5.43±0.33^{B}	4.28±0.30^{B}	9.7±0.2^{B}	35.4±1.5^{ab}	27.4±1.4^{b}

Continued

[1]Values are mean ± S.E for triplicate observations. Each triplicate consisted of liver samples pooled from 4 birds.

[2]Means not sharing a common superscript letter within a column are significantly different ($P<0.05$ or $P<0.01$).

[3]Units/hr/100 g BW is calculated by multiplying units/hr/g liver by the total liver weight by 100 and by dividing by body weight.

[4]ND: not determined.

Abbreviation: NFD, niacin free diet; BW, body weight.

Table 15. Continued.

Group/Diet		Aldolase			Malic enzyme		
		units/g liver	units/g liver protein	units/100 g BW	units/g liver	units/g liver protein	units/100 g BW[3]
I	NFD + 0.007% nicotinic acid (ad libitum)	100±10[a]	347±28[a]	308±12[a]	35.0±1.1[a]	121.7±5.2[a]	107.8±4.3[a]
II	NFD (ad libitum)	99± 6[a]	361±25[a]	332±28[a]	20.6±6.4[cb]	78.5±20.0[ab]	70.8± 24.4[ab]
III	NFD + 0.007% nicotinic acid (restricted)	115±11[a]	413±33[a]	287±23[a]	15.9±3.8[c]	58.0±5.0[b]	40.0±10.1[b]
IV	NFD + 0.66% tryptophan (ad libitum)	ND[4]	ND	ND	30.5±3.2[ab]	104.6±13.0[a]	93.0±6.7[a]
V	NFD + 0.035% nicotinic acid (ad libitum)	ND	ND	ND	28.7±1.6[ab]	102.5±6.1[a]	79.7±5.7[ab]

Continued

Table 15. Continued.

Group/Diet		Glutamic dehydrogenase			Lactic dehydrogenase		
		units/g liver	units/g liver protein	units/100 g BW	units/g liver	units/g liver protein	units/100 g BW
I	NFD + 0.007% nicotonic acid (ad libitum)	5.7±0.7[a]	18.5±1.1[a]	17.5±1.0[a]	409± 37[a]	1414±180[a]	1255±187[a]
II	NFD (ad libitum)	6.5±0.7[a]	20.9±1.5[a]	21.3±2.0[a]	301± 26[a]	1098± 91[a]	1007±123[a]
III	NFD + 0.007% nicotinic acid (restricted)	6.9±0.7[a]	21.7±1.6[a]	17.2±1.2[a]	423± 51[a]	1517±158[a]	1054± 85[a]
IV	NFD + 0.66% tryptophan (ad libitum)	ND[4]	ND	ND	ND	ND	ND
V	NFD + 0.035% nicotinic acid (ad libitum)	ND	ND	ND	ND	ND	ND

this enzyme increased during starvation. Tryptophan pyrrolase activity is also known to enhance in response to substrate induction (Feigelson and Greengard, 1962) and corticosteroid administration (Knox and Auerbach, 1955b). In addition, Schimke et al. (1965) reported that hydrocortisone administration increases the rate of tryptophan pyrrolase synthesis whereas tryptophan decreases the rate of degradation of the enzyme. They therefore suggested that hormone and substrate may play a different role in their mechanism of regulation of this enzyme in rat liver. The results in the current study also demonstrate that tryptophan pyrrolase activity did not increase in ad libitum fed quail which received a higher amount of tryptophan than the optimum requirement.

The activity of liver fructose diphosphatase, a non-pyridine nucleotide requiring enzyme, in the food-restricted group (III) was also significantly ($p < 0.01$) higher than that in the other four groups (I, II, IV, and V) when expressed as units/g liver. However, there were no significant differences in the levels of activity of liver fructose diphosphatase among the four groups (I, II, IV, and V). It was also of interest to note that there was some tendency for the liver fructose diphosphatase of the tryptophan fed group (IV) to decrease relative to the other three groups (I, II, and V). This may partially agree with the findings of Meijer et al. (1975) and Williamson et al. (1971) that tryptophan inhibits gluconeogenesis both in vivo and in vitro. If the activities of liver fructose diphosphatase were expressed as units per

g liver protein, a similar pattern also developed. However, if the activities of liver fructose diphosphatase were expressed as units per 100 g body weight, liver fructose diphohphatase activities were significantly ($p < 0.05$) different between group II and groups III, IV, and V, but not between group I and group II. These differences may be ascribed to a differential rate of tissue growth among the various treatment groups. Regarding the activities of gluconeogenic enzymes, Seitz et al. (1976) reported that fructose diphosphatase activity increased during starvation. This observation is in an accordance with observations in the current study when activities are expressed on a per unit liver weight. Overall these results would suggest that nicotinic acid deficiency did not affect the activity level of enzymes that are not directly involved in nicotinic acid metabolism but that feeding patterns had an influence on the activity level of these enzymes.

The activities of liver aldolase, lactic dehydrogenase and glutamic dehydrogenase were not affected by restricted feeding or by feeding a niacin free diet. Similarly, Garcia-Bunuel et al. (1962) also reported that there was no significant difference in the liver glutamic dehydrogenase activity between the niacin deficient and food-restricted rats, but that there was a slight increase in glutamic dehydrogenase activity of both niacin deficient and food-restricted rats as compared to the controls.

The activity of malic enzyme of the food-restricted

group (III) expressed as units per g liver was significantly ($p< 0.05$) lower than those of groups I, IV, and V although there were no significant differences between the niacin deficient (II) and the food-restricted (III) groups. Interestingly, the activity of malic enzyme of niacin deficient group (II) was also significantly ($p< 0.05$) lower than that of the control group (I) even though there were no significant differences in the activity of this enzyme among groups II, IV, and V. A similar trend also occurred when activity values were expressed as units per g liver protein or units per 100 g body weight. It may be concluded that the activity of malic enzyme in the two semi-starved groups [the niacin free group (II) and particularly the food-restricted group (III)] were much lower than those of groups I, IV, and V. Leveille (1969) also reported that starvation in chicks reduced the activity of malic enzyme in the liver. Overall, the results obtained with liver would suggest that the activity levels of both pyridine- and non-pyridine nucleotide requiring enzymes and an enzyme involved in tryptophan metabolism are not directly affected by the nicotinic acid status of the animal. However, some of these enzymes were influenced by the pattern of feeding such as restriction of feed intake.

The activity levels of two NAD requiring enzymes and one non-NAD requiring enzyme in breast muscle are presented in Table 16. No significant differences ($p> 0.05$) in the breast muscle lactic dehydrogenase or glyceraldehyde-3-phosphate dehydrogenase activity were observed between the niacin

Table 16. Effects of 5 Different Dietary Treatments on Lactic Dehydrogenase, Glyceraldehyde-3-phosphate Dehydrogenase and Aldolase in Breast Muscle Tissue of Japanese Quail (Experiment 6).[1,2]

Group/Diet	Lactic dehydrogenase		Glyceraldehyde-3-phosphate dehydrogenase		Aldolase	
	units/g muscle	units/g muscle protein	units/g muscle	units/g muscle protein	units/g muscle	units/g muscle protein
I NFD + 0.007% nicotinic acid (ad libitum)	1476±74^{a}	6184±354^{a}	224±17^{a}	938±69^{a}	188±11^{A}	785±32^{a}
II NFD (ad libitum)	989±135^{b}	4328±519^{b}	160±25^{b}	698±81^{b}	141±18^{AB}	618±51^{b}
III NFD + 0.007% nicotinic acid (restricted)	977±156^{b}	4424±555^{b}	114±24^{b}	653±79^{b}	100±16^{B}	455±53^{C}
IV NFD + 0.66% tryptophan (ad libitum)	1550±99^{a}	6202±409^{a}	243±20^{a}	967±68^{a}	199±2^{A}	801±50^{a}
V NFD + 0.035% nicotinic acid (ad libitum)	1560±94^{a}	6601±317^{a}	236±18^{a}	1000±69^{a}	201±5^{A}	856±47^{a}

[1] Values are mean ± S.E for triplicate observations. Each triplicate consisted of breast muscle samples pooled from 4 birds.

[2] Means not sharing a common superscript letter within a column are significantly different ($P<0.05$ or $P<0.01$).

Abbreviations: NFD, niacin free diet.

deficient (II) and food-restricted (III) groups, but there was a significantly ($p < 0.05$) higher lactic dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase activity levels in groups I, IV, and V as compared to groups II and III . Similarly, the activity of breast muscle aldolase of group III was significantly ($p < 0.01$) lower than that of groups I, IV, and V when expressed in units per g breast muscle. However, there were no significant differences in activity between groups II and III. Expressing activity values as units per g breast muscle protein yield similar results except that there are significant differences ($p < 0.05$) in the activity levels between groups II and III. The changes in lactic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and aldolase activities in the breast muscle can not be directly ascribed to the effect of niacin deficiency since similar changes also occurred in both the food-restricted group (III) and the niacin free group (II). These results would indicate that the decrease in the activity level of the dehydrogenases in these two groups relative to the control group (I) was directly influenced by calorie intake. The enzyme activity results are different from those for pyridine nucleotides. As indicated above, the niacin deficient group (II) was the only group that had a significantly lower level of NAD in breast muscle tissue. These results would indicate that the level of pyridine nucleotide in tissues may not be a significant factor influencing the concentration of the pyridine nucleotide requiring enzymes.

These observations would appear to be different from that observed with enzymes requiring other cofactors. For example, it has been well known that riboflavin deficiency in rats led to a marked lowering in the activities of FAD-dependent enzymes such as hepatic succinic dehydrogenase, α-glycerophosphate dehydrogenase , and xanthine oxidase (Zaman and Verwilghan, 1975). In addition, Katunuma et al. (1971) reported that rats with a pyridoxine deficiency showed a rapid decrease in the pyridoxal dependent enzymes, ornithine aminotransferase , serine dehydratase, tyrosine aminotransferase, and aspartate aminotransferase. They proposed that pyridoxine deficiency resulted in an increase in the activity of a group-specific protease which inactivates pyridoxal phosphate apoenzymes. Litwack and Rosenfield (1973) demonstrated that the rates of in vivo degradation of several pyridoxal enzymes correlate well with the in vitro dissociation of the enzyme into apoenzyme and pyridoxal phosphate. These findings have led to the concept that dissociation of coenzyme to yield unstable apoenzyme may be rate limiting for degradation of pyridoxal enzymes, differential rates of dissociation then being responsible for the observed heterogeneity of degradation rates in vivo.

In contrast, Lee et al. (1977) found that degradation rates are unaffected by pyridoxine deficiency when the loosely-bound pyridoxal phosphate dependent tyrosine aminotransferase and the tightly-bound pyridoxal phosphate dependent alanine aminotransferase were compared. From these

observations they concluded that coenzyme interaction with enzymes is not a significant determinant in intracellular degradation of the enzymes. Nevertheless, it would appear that the stability and the rate of degradation of pyridoxal phosphate dependent enzymes are influenced by the relative affinity for the pyridoxal phosphate and tissue coenzyme levels. It also seems reasonable to assume that these pyridoxal enzymes are readily attacked by a group-specific protease in the absence of the pyridoxal phosphate and as a result tend to have a relatively high degradation rate. A similar situation may also occur in the pyridine nucleotide dependent enzymes although the relative amounts of bound coenzymes may be considerably different.

Very little information is available on factors affecting the stability of pyridine nucleotide dependent enzymes. Katunuma et al. (1971) however reported that there is a group-specific protease for NAD- or NADP- dependent enzymes. It has been well documented that there are dissimilarities in the binding of NAD molecules to glyceraldehyde-3-phosphate dehydrogenase (Chance and Park, 1967) and the NAD-binding sites are not equivalent (Koshland and Neet, 1968). They also reported that the enzyme contains two moles of tightly bound NAD-1,2 ($K_{d1} = 10^{-11}$ M, $K_{d2} = 10^{-9}$ M) and two moles of relatively loosely bound NAD-3,4 ($K_{d3} = 3 \times 10^{-7}$ M, $K_{d4} = 2.6 \times 10^{-5}$ M) respectively. Takenaka and Schwert (1956) reported that the dissociation constant of LDH-NAD complex was found to be 3.9×10^{-4} M by ultracentri-

fugal separation measurements. The thermodynamic study of the binding of NAD to glyceraldehyde-3-phosphate dehydrogenase (DeVijlder et al., 1969) and lactic dehydrogenase (Subramanian and Ross, 1977; 1978) in rabbit muscle was reported. The $\Delta G°$ values for the binding of NAD to glyceraldehyde-3-phosphate dehydrogenase are -11 kcal for NAD-1,2, -9 kcal for NAD-3, and -6.6 kcal for NAD-4, respectively, whereas the calculated value for lactic dehydrogenase is -3.8 kcal mol^{-1} . The free energies of NAD binding to glyceraldehyde-3-phosphate dehydrogenase are more negative than that of NAD binding to LDH. These thermodynamic and kinetic studies indicate that the binding of NAD to glyceraldehyde-3-phosphate dehydrogenase is much stronger than that for LDH. In addition, Jaenicke (1969) reported that the rabbit muscle apoglyceraldehyde-3-phosphate dehydrogenase turned out to be extremely labile relative to the apolactic dehydrogenase. Ovadi et al. (1971) reported that glyceraldehyde-3-phosphate dehydrogenase exists as an equilibrium mixture of dimers and tetramers in solution and both forms are enzymically active. From these reports it can be speculated that the degree of saturation of dehydrogenases with NAD may vary depending on the availability of NAD in tissues and their relative affinity for the coenzymes.

It is conceivable that certain dehydrogenases may not be completely saturated with coenzymes under conditions of niacin deficiency. This effect would be attributable to the high affinity of dehydrogenase for coenzyme and to the

competition among dehydrogenases for the limited supply of available coenzymes. Those enzymes that have the lowest K_d values would selectively tend to bind coenzymes at the expense of those that have higher K_d values. Under these conditions it would be anticipated that the rate of degradation of the former enzymes would not be affected during niacin deficiency due to the possible stabilizing effects of the coenzyme whereas the enzymes with a low affinity for coenzymes would tend to be degraded at an accelerated rate. It can also be predicted that the amount of coenzymes relative to total binding sites of enzyme would determine the proportion of total dehydrogenases that are affected. Additional research will have to be carried out to more clearly establish the relationship between the affinity of dehydrogenase for pyridine nucleotides and their relative rates of degradation under conditions that exist in the cell.

It may be concluded that under conditions of severe niacin deficiencies there is a marked depression in growth, a differential rate of development, a reduction in food consumption, brittleness of the skull bone, blindness, and marked atrophy of the pectoral muscle. There is also a dramatic reduction in the level of NAD and NADPH in the pectoral muscle. Coenzyme levels in other tissues, however, are not affected by niacin deficiency. These observations would indicate that NAD(H) or NADP(H) may be transported from the breast muscle tissue to other essential organs to maintain the optimum level of pyridine nucleotides of these

tissues. These results also suggested that NADPH and particularly NAD levels in the breast muscle would provide the most reliable index of overall niacin status. NADPH levels in breast muscle also appear to be somewhat depressed in the niacin supplemented starved bird.

In the light of results with other enzymes requiring different coenzymes it was expected that there would also be a close association between tissue pyridine coenzyme levels and corresponding dehydrogenase activity levels . There was, however, no apparent relationship between the niacin status of quail and the corresponding activity levels of metabolically related dehydrogenases. In general, the breast muscle dehydrogenase activity levels of the niacin deficient quail were similar to that of the food-restricted quail whereas this relationship did not always occur with the liver enzymes. The different response in liver tissue, particularly with regard to malic enzyme, was attributed to a starvation effect rather than to an effect on niacin status of the animal. The activity levels of other metabolically related enzymes which are not pyridine nucleotide dependent were also not affected by niacin status in the animals . Tryptophan pyrrolase, an inducible enzyme involved in the conversion of tryptophan to nicotinic acid, was also not affected by niacin deficiency but by food restriction . Further research will be required to establish the relationship between niacin deficiency and the atrophy of breast muscle in relation to protein turnover.

PART IV. Studies on the Effect of Antiniacin (6-aminonicotinamide) on the Induction of Niacin Deficiency.

(A) Introduction

It has been established that certain structural analogs of nicotinic acid or nicotinamide interfere with the utilization of niacin for the synthesis or action of the pyridine coenzymes (Webb, 1966). In many instances the analogs have been found to produce a pattern of symptoms roughly similar to those seen in the niacin deficiency (Woolley 1945). Nevertheless, it should be clearly understood that the situations are basically different. A dietary restriction of niacin leading to a generalized depletion in the tissues would not necessarily bring about functional alterations identical to those caused by an analog, which could be much more effective in interfering with certain functions of the coenzyme than simple depletion and possibly leave other functions untouched.

The objective of this study was to determine if the mode of action of 6-aminonicotinamide (6-AN) was similar to that induced when quail were fed a niacin free diet. If both cause similar metabolic disturbances it should be possible to utilize 6-AN on a routine basis to investigate the effects of niacin deficiency. This would probably simplify the problems of inducing niacin deficiency in the mature quail (Part II, Experiment 2) and therefore would facilitate future studies. The present study was undertaken to obtain information concerning

the effect of 6-AN on growth, organ weights, activity levels of certain enzymes, and the content of pyridine nucleotides and ATP in liver and breast muscle tissues.

(B) Experimental Procedures

EXPERIMENT 7

(i) Design

Prior to the initiation of this experiment, mature male Japanese quail were provided with a mixed diet (chick starter diet plus niacin free diet) ad libitum for one week. During this adaptation period, the relative proportion of niacin free diet in a mixed diet was gradually increased. After this period, quail which weighed from 105 to 115 g, were divided into four groups of 25 quail each. Each group was fed one of the following diets: (i) niacin free diet plus 0.007% nicotinic acid, (ii) niacin free diet plus 0.007% nicotinic acid plus 0.014% 6-AN, (iii) niacin free diet plus 0.07% nicotinic acid plus 0.014% 6-AN, and (iv) niacin free diet plus 0.007% or 0.36% nicotinic acid plus 0.014 6-AN. In the case of diet 4, quail were fed the NFD, 0.007% nicotinic acid and 0.014% 6-AN for 10 days; then on day 10 all quail were switched to a new diet containing NFD, .36% nicotinic acid and 0.014% 6-AN. These quail remained on this latter diet for an additional 4 days.

All quail had free access to water and their respective diets for a period of two weeks. Body weights and mortalities were recorded daily or on alternative days throughout the ex-

perimental period. At the end of the experiment, all quail were sacrificed by decapitation and liver and breast muscle tissues were immediately removed, frozen in liquid nitrogen as described previously and stored at -70°C until analyzed. The Student's t-test was employed to estimate the significance of differences between two means (Hill, 1961).

(ii) Quantitative Procedures

The methods for the determination of enzyme activity levels and pyridine nucleotide concentrations were the same as described in General Experimental Procedures.

ATP was determined using the 3-phosphoglycerate kinase (ATP: 3-phospho-D-glycerate-1-phosphotransferase, EC.2.7.2.3) method (Jaworek et al., 1974). This assay involves the following reactions:

$$\text{glycerate-3-phosphate} + \text{ATP} \xrightleftharpoons{\text{PGK}} \text{glycerate-1, 3-P}_2 + \text{ADP} \quad \text{(i)}$$

$$\text{glycerate-1, 3-P}_2 + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{GAPdH}} \text{glyceraldehyde-3-phosphate} + \text{NAD}^+ + \text{Pi} \quad \text{(ii)}$$

Glycerate-1, 3-P_2 formed in reaction (i) was determined by the indicator reaction (ii) with glyceraldehyde-3-phosphate dehydrogenase. The oxidation of NADH, as measured by the change of extinction at 340 nm, is proportional to the amount of ATP present. All assays were carried out at 30°C in 1-cm cuvettes in a Gilford 2400 spectrophotometer. Duplicate or triplicate analyses were routinely performed.

(iii) Preparation of Tissue Extracts for ATP Determination

An appropriate portion of breast muscle tissue was homogenized in cold 0.9 M perchloric acid for 1.0 minute at the maximum speed of the Polytron homogenizer. The homogenates were pooled on an equal weight basis, centrifuged for 1.0 minute at 3,000 x g at 4°C and the supernatants were adjusted to pH 6.0 - 6.5 with cold 3.75 M K_2CO_3. The potassium perchlorate was removed by centrifugation and the extract was immediately used for ATP determination.

(C) Results and Discussion

Figure 14 illustrates the patterns of body weight loss when mature male quail were fed their respective diets. There were no changes in body weight for control quail throughout the experiment. However, the body weight of all 6-AN-treated quail declined very sharply from day 3 of the experiment regardless of the levels of nicotinic acid in diets included. After the quail were fed 6-AN for 14 days the weight loss of quail fed diets 2, 3 and 4 equalled 70.9, 80 and 68.8% of their original body weight respectively.

Although there were significant differences ($p < 0.001$) in body weight loss between the control and 6-AN-treated quail there were no significant differences ($P>0.05$) in body weight loss among three 6-AN-treated groups. In this experiment a 10 fold increase in the concentration of nicotinic acid (diet 3)

Figure 14. Growth Curves of Mature Male Japanese Quail as Affected by the Consumption of Nicotinic Acid Containing Diets Supplemented with or without 6-aminonicotinamide (Experiment 7).

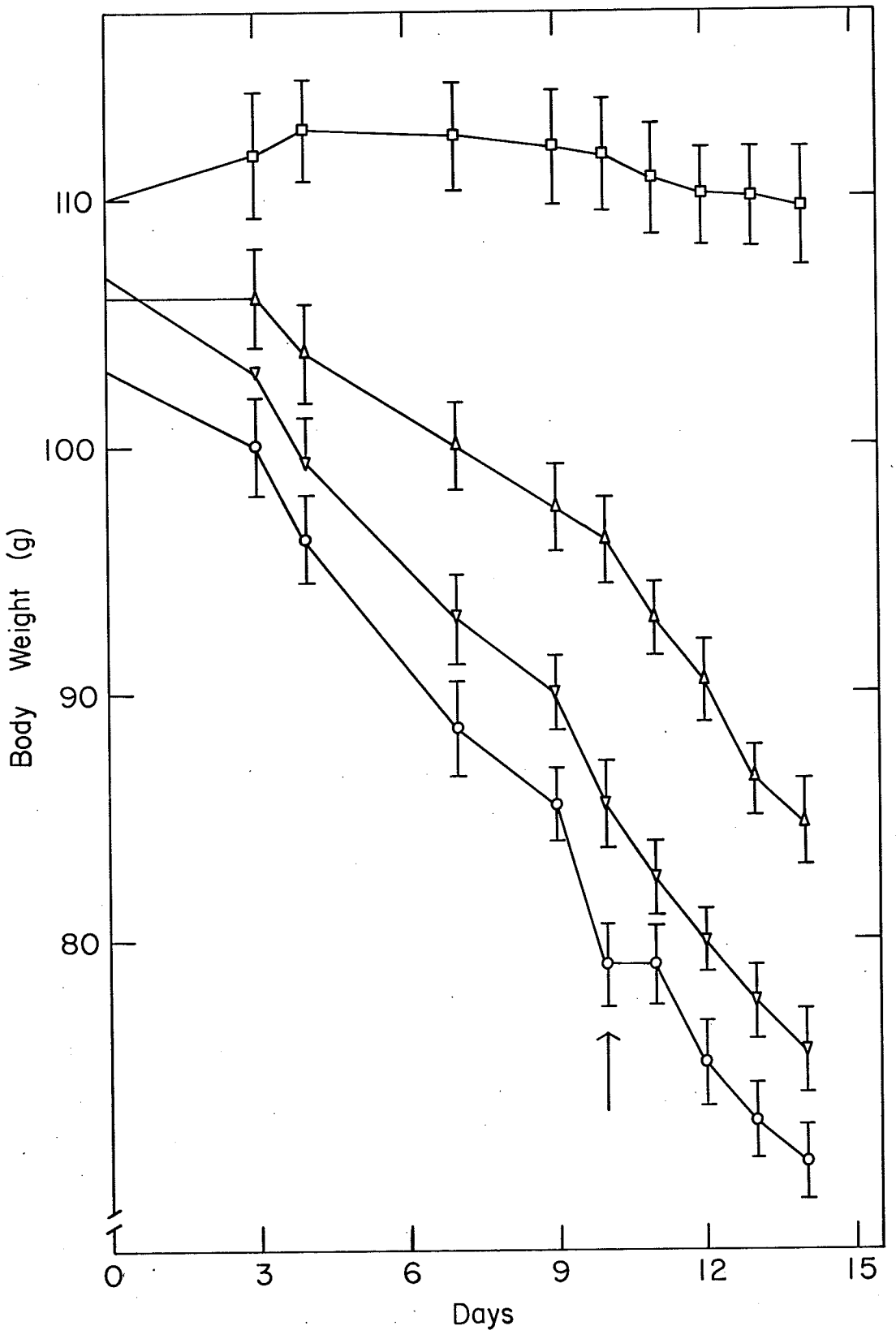
□ : NFD + 0.007% nicotinic acid (diet 1)

▽ : NFD + 0.007% nicotinic acid + 0.014% 6-aminonicotinamide (diet 2)

△ : NFD + 0.07% nicotinic acid + 0.014% 6-aminonicotinamide (diet 3)

○ : NFD + 0.007% nicotinic acid + 0.014% 6-aminonicotinamide (diet 4)

In the case of diet 4, quail were fed the above diet for 10 days after which they were given the same diet along with 0.36% nicotinic acid. Arrow indicates the addition of nicotinic acid to the diet.


110
100
90
80
Body Weight (g)
0
3
6
9
12
15
Days

did not alleviate the effects of antiniacin (diet 2). In a further effort to overcome the toxic effect by 6-AN, quail on diet 4 were fed the NFD that contained 0.36% nicotinic acid and 0.014% 6-AN on the day 10th of the experiment. As indicated in Figure 14, the relatively high levels of dietary nicotinic acid also failed to alleviate body weight losses.

In addition to body weight loss and reduced food intake, most of 6-AN-treated quail appeared to demonstrate some signs of niacin deficiency such as poor feathering and lack of mobility throughout the course of the experiment. Similar observations were also made by Johnson and McColl (1955) who reported the loss of motor control and paralysis several hours after the injection of a single dose of 6-AN to rats. In an experiment similar to Johnson and McColl (1955), Lison (1970) pointed out that 6-AN lowers the body temperature, increases the blood sugar concentration, and causes a loss of body weight. It was also reported that the decline of body weight following the injection of 6-AN (5 mg per kg body weight) resembles that seen after starvation.

In the current study no mortalities were observed with the control quail whereas the number of mortalities in quail fed diets 2, 3, and 4 were 9, 12, and 14 respectively. It may be concluded that the administration of small quantities of 6-AN resulted in a very pronounced effect on body metabolism as indicated by the marked loss of body weight, lack of mobility and higher incidence of mortality.

Control quail and the quail fed 0.007% nicotinic acid and 0.014% 6-AN (diet 2) were chosen to further investigate effects of 6-AN on biochemical parameters. The weights of breast muscle and heart tissues of control quail were significantly ($P<0.001$) higher than those of the 6-AN-treated group whereas liver weights were oppositely affected ($P<0.001$), (Table 17). The color of liver in control quail was reddish while that of 6-AN-treated quail was yellowish. This must be due to the toxic effects of 6-AN on liver tissue. The effects of 6-AN on heart and breast muscle mass but not liver was similar to those obtained in the previous study (Part III, Experiment 6) with the niacin deficient quail.

As shown in Tables 19 and 20, there were no significant differences in the concentration of protein in breast muscle and liver between the control and 6-AN-treated quail.

The effect of 6-AN on ATP contents of breast muscle tissue was also examined (Table 18). There were also no significant differences ($P>0.05$) between these two groups in the concentration of ATP in breast muscle tissue. A similar observation was also made by Lange *et al* (1970) who reported that 6-AN did not change the ATP level in the brain. In contrast, Kauffman and Johnson (1974) reported that levels of ATP were reduced approximately 12 percent in brains of mice treated with 6-AN as compared to normal control levels. Similarly, Dietrich *et al* (1958 a) also observed that there was a pronounced reduction in the levels of ATP and ADP in

Table 17. Effect of 6-Aminonicotinamide on Body Weight Loss, Total Liver Weight, Breast Muscle Weight, Heart Weight, and Mortality (Experiment 7). [1,2]

Diet	Initial BW (g)	Final BW (g)	BW loss (g)	Liver WT (g)	Breast Muscle WT (g)	Heart WT (g)	No. of Mortality
NFD + 0.007% nicotinic acid	110±3	109±3	1±0.5	1.65±0.07	20.7±0.6	0.98±0.03	0
NFD + 0.007% nicotinic acid + 0.014% 6-amino-nicotinamide	109±2	76±2	32±2.5	2.35±0.13	11.2±0.6	0.71±0.02	9
	P>0.05	P<0.05	P<0.001	P<0.001	P<0.001	P<0.001	

[1]Values are mean ± S. E. of 15 observations.

[2]Student's t-test was used to calculate the level of significance between two means.

Abbreviations: BW, body weight; WT, weight; NO, number.

Table 18. Liver NAD Level, Breast Muscle Pyridine Coenzyme and ATP Levels of Quail Fed Nicotinic Acid and 6-Aminonicotinamide (Experiment 7)[1].

Diet	N A D					
	Liver			Breast Muscle		
	µmoles/g liver	µmoles/g liver protein	µmoles/100 g BW[2]	µmoles/g muscle	µmoles/g muscle protein	µmoles/100 g BW
NFD + 0.007% nicotinic acid	0.65±0.06	2.24±0.23	1.00±0.14	0.87±0.03	2.48±0.10	16.54±0.85
NFD + 0.007% nicotinic acid + 0.014% 6-amino-nicotinamide	0.76±0.02	2.44±0.12	2.38±0.21	0.85±0.04	2.42±0.10	12.95±0.52
	P>0.05	P>0.05	P<0.001	P>0.05	P>0.05	P<0.01

[1]Values are mean ± S.E for quadruple observations. Each quadruple consisted of liver or breast muscle samples pooled from 3 birds.

[2]µmoles/100 g BW are calculated by multiplying µmoles/ g liver or g breast muscle by multiplying the total liver or breast muscle weights by 100 and by dividing by body weight.

Student's t-test was used to calculate the level of significance between two means.

Abbreviation: BW, body weight.

Table 18. Continued

Diet	N A D P			A T P		
	µmoles/g muscle	µmoles/g muscle protein	µmoles/100 g BW	µmoles/g muscle	µmoles/g muscle protein	µmoles/100 g BW
NFD + 0.007% nicotinic acid	0.018±0.004	0.051±0.009	0.974±0.03	5.87±0.88	16.70±0.62	111.4±19.0
NFD + 0.007% nicotinic acid + 0.014% 6-aminonicotin-amide	0.016±0.003	0.045±0.007	0.672±0.02	6.36±0.21	18.20±0.71	9.37±7.9
	P>0.05	P>0.05	P<0.01	P>0.05	P>0.05	P>0.05

the tumor tissue treated with 6-AN. These results would suggest that the effect of 6-AN on the ATP levels has not been clearly established.

There were no significant differences ($P>0.05$) in levels of breast muscle NAD and NADP between control and 6-AN-treated quail whether the levels were expressed in μmoles/g breast muscle or μmoles/g breast muscle protein. However, if the levels of these pyridine coenzymes were expressed in μmoles/100 g body weight, the levels of NAD and NADP of control quail were significantly ($P<0.01$) higher than the corresponding levels in the 6-AN-treated quail (Table 18).

Similarly, no significant differences were observed in the levels of liver NAD between control and 6-AN-treated quail. However, the liver NAD contents per 100 g body weight of 6-AN treated quail were significantly ($P<0.001$) higher than the corresponding control values. This can be attributed to the larger size of liver weight of 6-AN-treated quail. These results would indicate that 6-AN did not exert a specific effect on tissue concentrations of these nucleotides.

In support of the current research Coper et al (1966) reported that total NAD and NADP levels did not change in liver and brain of mice exposed to 6-AN. They postulated that some mechanisms must exist which maintained pyridine nucleotides at certain optimum levels in mammalian tissue. In contrast, Dietrich et al (1958b) reported that NADP in the liver and kidney of mice was converted to 6-ANADP (6-aminonicotinamide adenine dinucleotide phosphate) by 6-AN, resulting in a decrease of NADP.

The data on liver and breast muscle enzyme activities are summarized in Tables 19 and 20. There was no significant difference ($P>0.05$) in specific activities of liver glyceraldehyde-3-phosphate dehydrogenase and lactic dehydrogenase between control and 6-AN-treated quail if activities expressed in units/g liver or units/g liver protein. However, the activities of these enzymes when expressed in terms of units/100 g body weight were significantly ($P<0.001$, $P<0.005$) higher in the 6-AN-treated quail as compared to those in the control quail. These observations would suggest that the increased activities of these enzymes were mainly due to the larger liver size of the 6-AN-treated group as compared to the control group. Interestingly enough, activities or specific activities of liver malic enzyme ($P<0.001$) and fructose diphosphatase ($P<0.05$, $P<0.001$) of the control quail were significantly higher than those of the 6-AN-treated quail when activities expressed in units/g liver or units/g liver protein. The control quail also had a higher malic enzyme activity ($P<0.05$) but a lower fructose diphosphatase activity ($P<0.001$) than the 6-AN-treated quail when activity values were expressed as units/100 g body weight.

In the case of breast muscle enzymes, no significant differences were found in activities or specific activities of LDH, glyceraldehyde-3-phosphate dehydrogenase, and aldolase between control and 6-AN-treated quail when activities were expressed in units/g muscle or units/g muscle protein. However, if activities were expressed in units/100 g body weight, the activities of breast muscle LDH ($P<0.05$) and

Table 19. Liver Protein Level and Enzyme Activities of Quail Fed Nicotinic Acid and 6-Aminonicotinamide (Experiment 7).[1]

Diet		Malic enzyme			Glyceraldehyde-3-phosphate dehydrogenase		
	g protein/g liver	units/g liver	units/g liver protein	units/100 g BW[2]	units/g liver	units/g liver protein	units/100 g BW
NFD + 0.007% nicotinic acid	0.29± 0	29.0±2.3	99.3±9.3	44.3±5.1	109± 6	372±20	166±13
NFD + 0.007% nicotinic acid + 0.014% 6-amino-nicotinamide	0.31± 0	9.0±1.7	29.2±6.3	27.6±4.6	107± 5	343±19	330±10
	P>0.05	P<0.001	P<0.001	P<0.05	P>0.05	P>0.05	P<0.001
		Lactic dehydrogenase			Fructose diphosphatase		
NFD + 0.007% nicotinic acid		657±57	2240±183	996±99	17.3±0.7	58.8±0.9	26.1±1.3
NFD + 0.007% nicotinic acid + 0.014% 6-amino-nicotinamide		565±14	1814± 24	1771±140	14.4±0.8	46.3±2.0	44.7±2.0
		P>0.05	P>0.05	P<0.005	P<0.05	P<0.001	P<0.001

[1]Values are mean ± S.E for quadruple observations. Each quadruple consisted of liver samples pooled from 3 birds.

[2]Units/100 g BW are calculated by multiplying units/g liver by multiplying the total liver weight by 100 and by dividing by body weight.

Student's t-test was used to calculate the level of significance between two means.

Abbreviation: BW, body weight.

Table 20. Breast Muscle Protein Level and Enzyme Activities of Quail Fed Nicotinic Acid and 6-Aminonicotinamide (Experiment 7).[1]

Diet	Protein	Glyceraldehyde-3-phosphate dehydrogenase			Lactic dehydrogenase			Aldolase		
	g protein/g liver	units/g muscle	units/g muscle protein	units/100 g BW[2]	units/g muscle	units/g muscle protein	units/100 g BW	units/g muscle	units/g muscle protein	units/100 g BW
NFD + 0.007% nicotinic acid	0.35±0	118± 7	337±21	2236±82	1213±88	3467±266	22921±1081	149±11	425±34	2812 157
NFD + 0.007% nicotinic acid + 0.014% 6-amino-nicotinamide	0.35±0	117±10 P>0.05	335±31 P>0.05	1814±220 P>0.05	1156±72 P>0.05	3302±232 P>0.05	17858±1748 P<0.05	129±4 P>0.05	367±17 P>0.005	1980 146 P<0.005

[1] Values are mean ± S.E for quadruple observations. Each quadruple consisted of breast muscle samples pooled from 3 birds.

[2] Units/100 g BW are calculated by multiplying units/g liver by multiplying the total breast muscle weight by 100 and by dividing by body weight.

Student's t-test was used to calculate the level of significance between two means.

Abbreviation: BW, body weight.

aldolase ($P<0.005$) of the control quail were significantly higher than those of 6-AN-treated quail. The higher activities of the enzymes in the control quail also were mainly attributed to the larger size of the breast muscle. No significant difference ($P>0.05$) was detected in the activity of breast muscle glyceraldehyde-3-phosphate dehydrogenase activity (units/100 g body weight) between control and 6-AN-treated quail.

With respect to the effect of 6-AN on NAD- or NADP-dependent enzymes, Friedland et al (1958) reported that the activities of pyruvic and lactic dehydrogenases of tumor cells were not affected by 6-AN. In contrast, Herken et al (1969) reported that brain 6-phosphogluconate dehydrogenase was markedly inhibited in 6-AN-treated rats.

In conclusion, the change in the concentration of NAD in breast muscle and liver tissues in the 6-AN-treated quail were quite different from that observed for niacin deficient quail in the previous experiment (Part III, Experiment 6). The concentration of NAD in breast muscle of niacin deficient quail was only 36% of that of the corresponding control value whereas no significant change was observed in 6-AN-treated quail as compared to control quail. However, the concentration of NAD in the liver of the niacin deficient birds (Part III, Experiment 6) and in the 6-AN treated birds in the current experiment were similar to control fed birds.

The patterns of change in enzyme activity levels were also different between 6-AN-treated and niacin deficient quail. In 6-AN-treated quail liver fructose diphosphatase (units/g

liver) showed much lower activity relative to control quail ($P<0.05$) whereas there were no significant differences ($P>0.05$) in the levels of this enzyme activity between niacin deficient and control quail. In the case of breast muscle enzymes, the levels of LDH and glyceraldehyde-3-phosphate dehydrogenase activities (units/g muscle) in niacin deficient quail were significantly ($P<0.05$) lower than those in control quail whereas there were no significant ($P>0.05$) differences between 6-AN-treated and control quail.

These observations therefore indicate that the effect of 6-AN on animal metabolism appears to be different from those obtained in the niacin deficient animal. It can be concluded that 6-AN should not be used in an attempt to duplicate physiological conditions similar to those induced by niacin deficiency. Additional research will have to be carried out to more clearly establish the extent of these differences.

PART V. The Fate of 7-^{14}C-nicotinic acid in Different Tissues of Japanese Quail fed a Niacin Free Diet and Restricted Amount of a Niacin Supplemented Diet.

(A) Introduction

It has been well established that 7-^{14}C-nicotinic acid is converted by human erythrocytes and rat liver to nicotinic acid mononucleotide and nicotinic acid-adenine dinucleotide and that these two nucleotides are intermediates in the biosynthesis of NAD from nicotinic acid (Preiss and Handler, 1958).

Although the biosynthesis of pyridine nucleotides has been extensively explored under normal metabolic conditions, little or no information is available concerning the metabolic fate of 7-^{14}C-nicotinic acid in different tissues under niacin deficiency and semi-starvation conditions. Also, little or no information except for that reported by Gerber and Deroo (1969) is available on turnover rates of niacin in animal tissues. These authors investigated the metabolism of NAD in irradiated mice and rats. They found that labelled NAD is conserved in some organs for a considerable length of time and that differences exist between organs with respect to incorporation of precursors into NAD.

The objective of this experiment was to compare the turnover rates of niacin metabolites in the liver, heart and breast muscle tissues of semi-starved and niacin deficient quail. This information will be obtained from half-life

values of the radioactive products (nicotinamide, NAD and NADP) that are formed following the administration of 7-^{14}C-nicotinic acid.

(B) Experimental Procedures

EXPERIMENT 8

(i) Management of Quail

Newly hatched quail were fed a mixture of the niacin free diet and a commercial chick starter diet (Feed-Rite Ltd.) until they reached 20 g. During this adaptation period, the relative proportion of niacin free diet in a mixed diet was gradually increased. After this period, the quail were then divided into two groups of from 45 to 50 quail each and were fed either the niacin free diet ad libitum or restricted amounts of the nicotinic acid supplemented diet. The amount of feed provided to this latter group was sufficient to maintain body weight gains at the same level as those fed the niacin free diet.

All quail were housed in electrically heated stainless steel batteries with wire floors. Quail weights were determined daily throughout the course of the experiment. After four days on treatment, the quail fed the NFD showed signs of niacin deficiency as manifested by a poor growth. They were maintained on this diet for an additional period of 4 days so as to further deplete their niacin reserves. On day 8, the quail were fasted

for 7 hours and 1.84 μCi (30 mμmoles) of 7-^{14}C-nicotinic acid (specific activity of 61 mCi/mmole) was injected intraperitoneally into each bird from the two groups. After injection, the four quail from each group were sacrificed at various time intervals: 5 hours, 13 hours, 25 hours, 50 hours, 74 hours, 99 hours, 127 hours, 172 hours, 221 hours and 275 hours. The liver, heart and breast muscle were rapidly removed, blotted free of blood, frozen in liquid nitrogen and stored at -70°C until analyzed.

(ii) Preparation of Tissue Samples

An appropriate portion of liver, heart and breast muscle was weighed and homogenized in cold 0.6 N $HClO_4$ for 1.0 minute with a Polytron homogenizer. Homogenates from two quail were pooled on an equal weight basis in a manner similar to that of Gerber and Deroo (1970). The homogenate was then centrifuged at 12,000 x g for 10 minutes. After centrifugation, 0.1 or 0.2 ml aliquots of the protein-free supernatant was pipetted into scintillation vials for counting.

(iii) Separation of Radioactive Products by DEAE Cellulose Column Chromatography

An adequate portion of liver and breast muscle was weighed and homogenized in cold 3% $HClO_4$ for 1.0 minute using a Polytron homogenizer. Samples from two quail were pooled on an equal weight basis and the homogenates were centrifuged

at 12,000 x g for 10 minutes. The protein-free supernatant was placed in an ice water bath and the pH was adjusted to 6.5 to 7.0 with 3 N KOH (Ijichi et al, 1966). After potassium perchlorate was removed by centrifuging, 2-3.0 ml aliquots of the radioactive supernatant solution containing authentic compounds was applied to a DEAE cellulose column (1.2 x 20 cm). The column was previously equilibrated with distilled water.

Authentic compounds were added to the extracts to improve the resolution of the radioactive peaks and to serve as markers. They were nicotinamide (2.0 μmoles), nicotinamide mononucleotide (2.0 μmoles), nicotinic acid (10 μmoles), NAD (2.0 μmoles), nicotinic acid mononucleotide (2.0 μmoles), deamido-NAD (2.0 μmoles) and NADP (2.0 μmoles). After sample application and thorough rinsing with distilled water, elution was carried out with a linear gradient of from 0 to 0.4 M NH_4HCO_3. The eluting buffer consisted of 450 ml of distilled water in the mixing chamber and 450 ml of 0.4 M NH_4HCO_3 in the reservoir (Chaykin et al, 1965). Fractions (5 ml) were collected at the rate of about 80 ml per hour. One milliliter aliquots were drawn for radioactive counting and the remaining portions were used for the determination of absorption at 260 nm. Peaks were identified on the basis of cochromatography of pure compounds (Chaykin et al, 1965). The identity of NAD and NADP peaks were further confirmed by the use of pyridine nucleotide assays (see General Experimental Procedures). Half-lives were calculated by least squares regression analysis (Dixon and Brown, 1977) from the equation $t_{\frac{1}{2}} = 0.693/k$, where k is the

slope of the first order activity decay curve.

(iv) Radioactive Counting

All samples were suspended in 10 ml of scintillation solvent and refrigerated at $4^{o}C$ in the dark for at least 24 hours so as to minimize chemiluminescence prior to counting. The scintillation solvent contained 16.5 g of 2,5-diphenyl-oxazole (PPO) and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP) dissolved in 2 ℓ of toluene and 1 ℓ of Triton X-1000. The samples were counted in Nuclear Chicago Mark II Liquid Scintillation Counter using sample channel ratio method to correct for quenching.

(C) Results and Discussion

Growth rates were greatly decreased (Figure 15) and feathering patterns were abnormal when the average body weights of the niacin deficient group reached approximately 30 g on day 4 after the initiation of test. From day 4 to day 15 there was little or no change in body weights of the group fed the NFD. Birds in the 17 to 19 days treatment groups appeared to gain weight at a slow rate. These latter weights however may not be a precise indicator of overall weights as they represent a progressively decreasing number of quail. The pattern of weight change in the two groups was nearly identical for both groups.

The data from Table 21 show that body, liver, heart and breast muscle weights of the niacin deficient and restricted

Figure 15. Growth Curves of Quail Fed the Nicotinic Acid Free Diet (o——o) and Restricted Amount of the Nicotinic Acid Supplemented Diet (□———□) (Experiment 8).

Each point represents the mean of a progressively decreasing number of quail (45 or 50 to 4) and the bars represent standard errors. Four quail were withdrawn for sacrifice at designated time intervals as indicated by arrows on the graph.

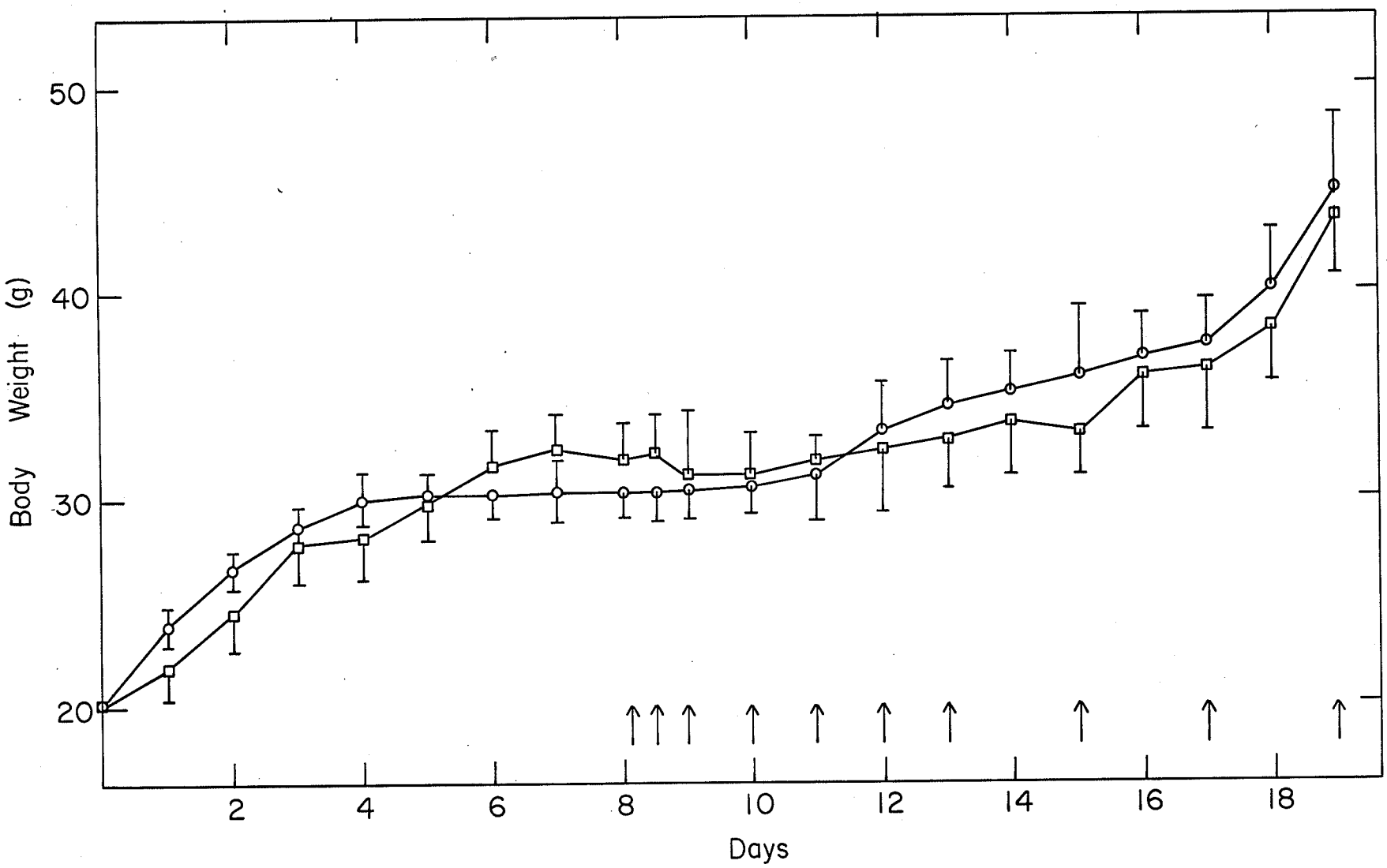

Body Weight (g)
50
40
30
20
2
4
6
8
10
12
14
16
18
Days

Table 21. Effects of Niacin Deficiency and Food Restriction on Body, Liver, Heart and Breast Muscle Weights Following the Administration of 7-^{14}C-Nicotinic Acid. (Experiment 8).[1]

Time after isotope administration	NFD, ad libitum				NFD + 0.007% nicotinic acid restricted			
	Body wt	Liver wt	Heart wt	Breast Muscle wt	Body wt	Liver wt	Heart wt	Breast Muscle wt
hour	g	g	g	g	g	g	g	g
5	30.2±0.8	0.92±0.03	0.21±0.01	3.66±0.20	32.0±1.1	1.11±0.05	0.22±0.00	3.84±0.22
13	30.5±1.2	0.93±0.08	0.20±0.01	3.50±0.19	32.2±2.0	1.03±0.08	0.24±0.00	3.73±0.17
25	30.7±1.2	0.98±0.06	0.22±0.03	3.56±0.28	31.5±3.0	1.04±0.10	0.23±0.01	3.79±0.20
50	30.8±0.8	1.32±0.05	0.23±0.01	3.72±0.25	31.6±2.2	1.53±0.10	0.23±0.02	3.84±0.30
74	31.3±2.8	1.09±0.12	0.24±0.02	4.00±0.35	31.5±1.1	0.93±0.02	0.24±0.01	3.90±0.31
99	33.2±2.7	1.21±0.15	0.25±0.01	4.09±0.37	32.2±3.0	0.97±0.07	0.23±0.03	4.04±0.38
127	34.6±2.2	1.09±0.04	0.25±0.01	4.12±0.40	33.0±2.8	1.30±0.08	0.26±0.01	4.05±0.35
172	36.5±3.1	1.36±0.15	0.27±0.01	4.46±0.42	33.1±2.2	1.09±0.13	0.26±0.02	4.32±0.38
221	37.1±2.8	1.58±0.17	0.29±0.03	4.78±0.48	38.0±3.0	1.31±0.14	0.27±0.01	4.63±0.44
275	45.0±3.2	1.30±0.05	0.30±0.01	5.28±0.51	43.3±3.0	1.69±0.34	0.27±0.01	5.53±0.54

[1]Values are mean ± S. E of 4 birds.
No between treatment differences were observed among the tissues (P>0.05).
Abbreviations: NFD, niacin free diet; wt, weight.

groups were not significantly different ($P>0.05$). Therefore, any differences in labelling patterns between the two groups can not be attributed to difference in growth rates.

The data presented in Table 22 and the analysis of variance data (Table 23) demonstrate that tissue radioactivity levels were dependent upon diet, type of tissue and time after administration of isotope. Initially the highest radioactivity levels for both groups occurred in the liver followed by heart and breast muscle tissues. This latter effect is illustrated by the observation that 5 hours after the injection the relative radioactivity in the liver of the food-restricted group was 13.0 and 44.0 fold greater than that of heart and breast muscle respectively. The values for the niacin deficient group followed the same trend but were higher. They were 19 and 104, respectively during the same time period. These results not only demonstrate that there is a difference in the relative concentration of label among tissues but also suggest that a lower proportion of the total radioactivity was transferred to the heart and particularly the breast muscle in the niacin deficient quail as compared to that for the food-restricted group. This trend was slightly modified during the time course of the study. This is exemplified by the fact that 5 hours after the injection 6% and 10.4% of the total label was associated with the heart plus breast muscle tissues in the niacin deficient and food-restricted quail respectively. In contrast, the corresponding values increased after 275 hours to 55% in the niacin deficient quail and to 77% in the food-restricted quail.

Table 22. Incorporation of 7-^{14}C-Nicotinic Acid into Different Organs of Quail at Various Time Intervals (Experiment 8)[1].

Tissue	Activity [(dpm/g of tissue)/(dpm injected/g of body weight)]					
	5 hour	13 hour	25 hour	50 hour	74 hour	99 hour
NFD (ad libitum)						
Liver	10.41±0.40	5.90±0.68	6.14±0.35	5.50±0.51	4.71±0.21	4.19±0.17
Heart	0.56±0.03	0.54±0.05	0.68±0.05	0.84±0.06	0.93±0.13	1.09±0.03
Breast Muscle	0.10±0.00	0.09±0.00	0.14±0.00	0.16±0.00	0.12±0.01	0.14±0.00
NFD + 0.007% nicotinic acid (restricted)						
Liver	7.44±0.21	3.81±0.31	4.68±0.85	2.77±0.20	2.00±0.14	1.89±0.06
Heart	0.59±0.00	0.55±0.02	0.69±0.03	0.77±0.03	0.87±0.02	0.90±0.04
Breast Muscle	0.17±0.02	0.21±0.03	0.28±0.03	0.56±0.04	0.51±0.00	0.63±0.02

Continued

[1]Values are mean ± S.E for duplicate observations. Each duplicate consisted of tissue samples pooled from 2 birds.
Abbreviation: NFD, niacin free diet.

Table 22. Continued

Tissue	Activity [(dpm/g of tissue)/(dpm injected/g of body weight)]			
	127 hour	172 hour	221 hour	275 hour
NFD (ad libitum)				
Liver	4.0±0.40	1.81±0.22	1.79±0.02	0.92±0.00
Heart	1.01±0.03	1.01±0.01	1.09±0.09	0.98±0.05
Breast Muscle	0.13±0.00	0.14±0.00	0.15±0.00	0.14±0.01
NFD + 0.007% nicotinic acid (restricted)				
Liver	0.73±0.07	0.54±0.03	0.30±0.01	0.18±0.01
Heart	0.65±0.08	0.52±0.00	0.44±0.18	0.29±0.00
Breast Muscle	0.47±0.03	0.48±0.05	0.38±0.06	0.31±0.06

Table 23. Incorporation of 7-^{14}C-Nicotinic Acid into Different Organs of Quail at Various Time Intervals.

Analysis of Variance (Table 22, Continued)

Source of Variation	df	Sum of Square	Mean Square	F
Diet	1	14.5160	14.5160	191.16**
Tissue	2	240.7095	120.3548	1584.95**
Time	9	70.3101	7.8122	102.88**
Diet x Tissue	2	30.9879	15.4939	204.04**
Diet x Time	9	1.32P7	0.1477	1.95
Tissue x Time	18	158.5555	8.8086	116.00**
Diet x Tissue x Time	18	5.7858	0.3214	4.23**
Error	60	4.5562	0.0759	
Total	119	525.7471		

**Significant at $P<0.01$.

Abbreviation: df, degree of freedom.

There was also a progressive increase in the total portion of the label in heart and breast muscle tissues up to 99 hours for both the niacin deficient and food-restricted groups. After 127 hours the corresponding values remained unchanged for the niacin deficient group whereas the values for the food-restricted group progressively declined during the remainder of the study. These differential patterns of change in the relative radioactivity for these two groups may be attributed to differences in the degree of conservation of the label in the heart and breast muscle relative to the liver, to a selective transfer of the label from the liver to these tissues or to the excretion of the label.

These results would suggest that the replacement of these radioactive products in liver, heart and breast muscle of the niacin deficient quail seems to be much slower relative to that of the food-restricted quail. It is also clear that the replacement of these radioactive products in different tissues was found to be most rapid in liver, followed by heart and slowest in the breast muscle for both the niacin deficient and food-restricted groups.

Plots of total radioactivity recovered in tissues from the two groups of birds at various times after the administration of 7-^{14}C-nicotinic acid are illustrated in Figures 16-18. The half lives of the radioactive products formed from the injection of 7-^{14}C-nicotinic acid were calculated by the use of time intervals which represented the linear portion of the different curves. As shown in Figures 16-18 the pattern of decline in radioactivity appeared to be multiphasic in nature.

Figure 16. Plots of Total Radioactivity in Liver versus Time after Injection of 7-^{14}C-Nicotinic Acid for Quail Fed either the Nicotinic Acid Free Diet (□——□) or Restricted Amounts of the Nicotinic Acid Supplemented Diet (o——o) (Experiment 8).

Each point represents the mean of duplicate observations. Each duplicate consisted of liver samples pooled from two quail each.

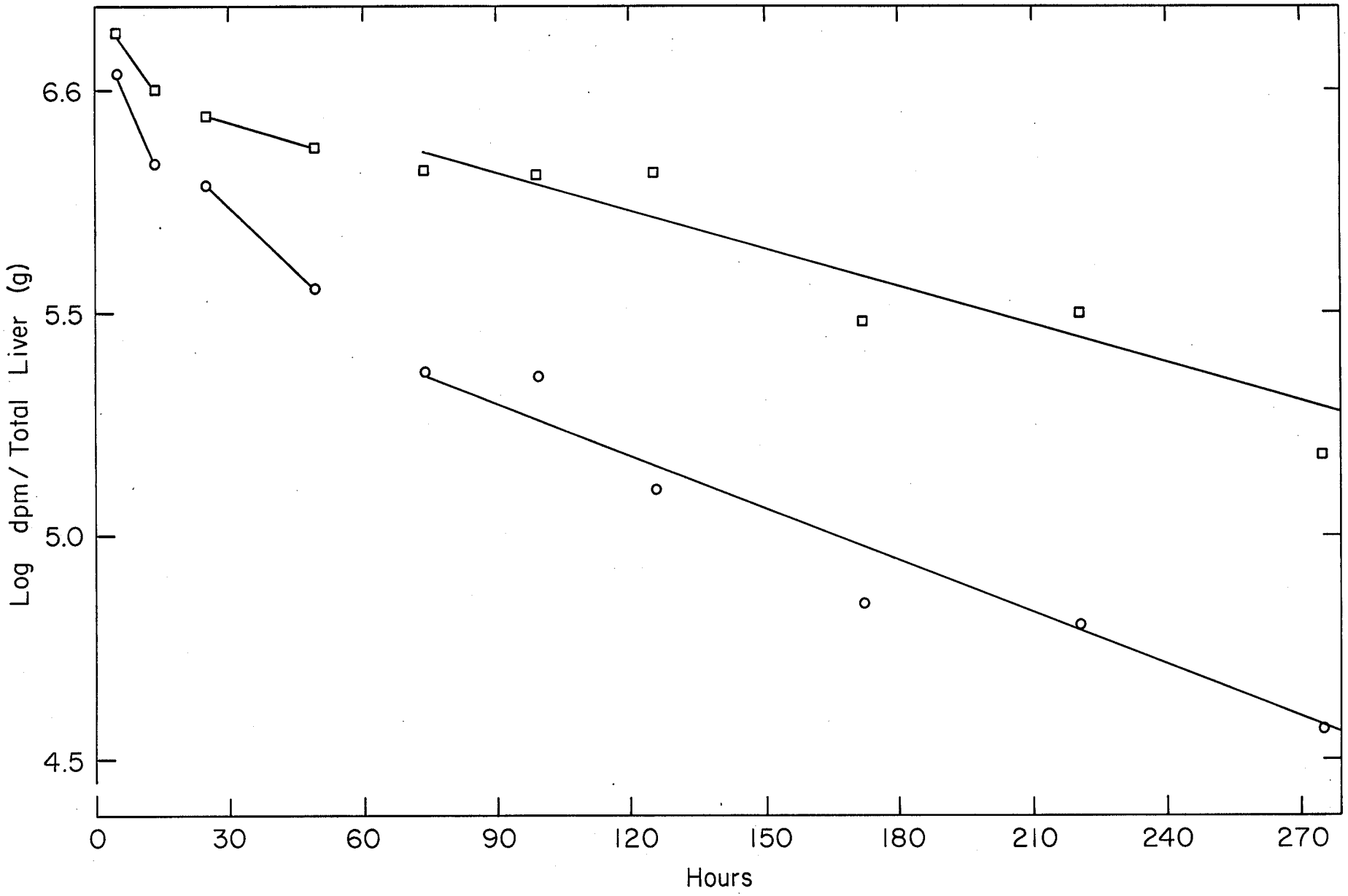

6.6
5.5
5.0
4.5
Log dpm/ Total Liver (g)
0
30
60
90
120
150
180
210
240
270
Hours

Figure 17. Plots of Total Radioactivity in Heart versus Time after Injection of 7-^{14}C-Nicotinic Acid for Quail Fed either the Nicotinic Acid Free Diet (□——□) or Restricted Amounts of the Nicotinic Acid Supplemented Diet (o——o) (Experiment 8).

Each point represents the mean of duplicate observations. Each duplicate consisted of heart samples pooled from two quail each.

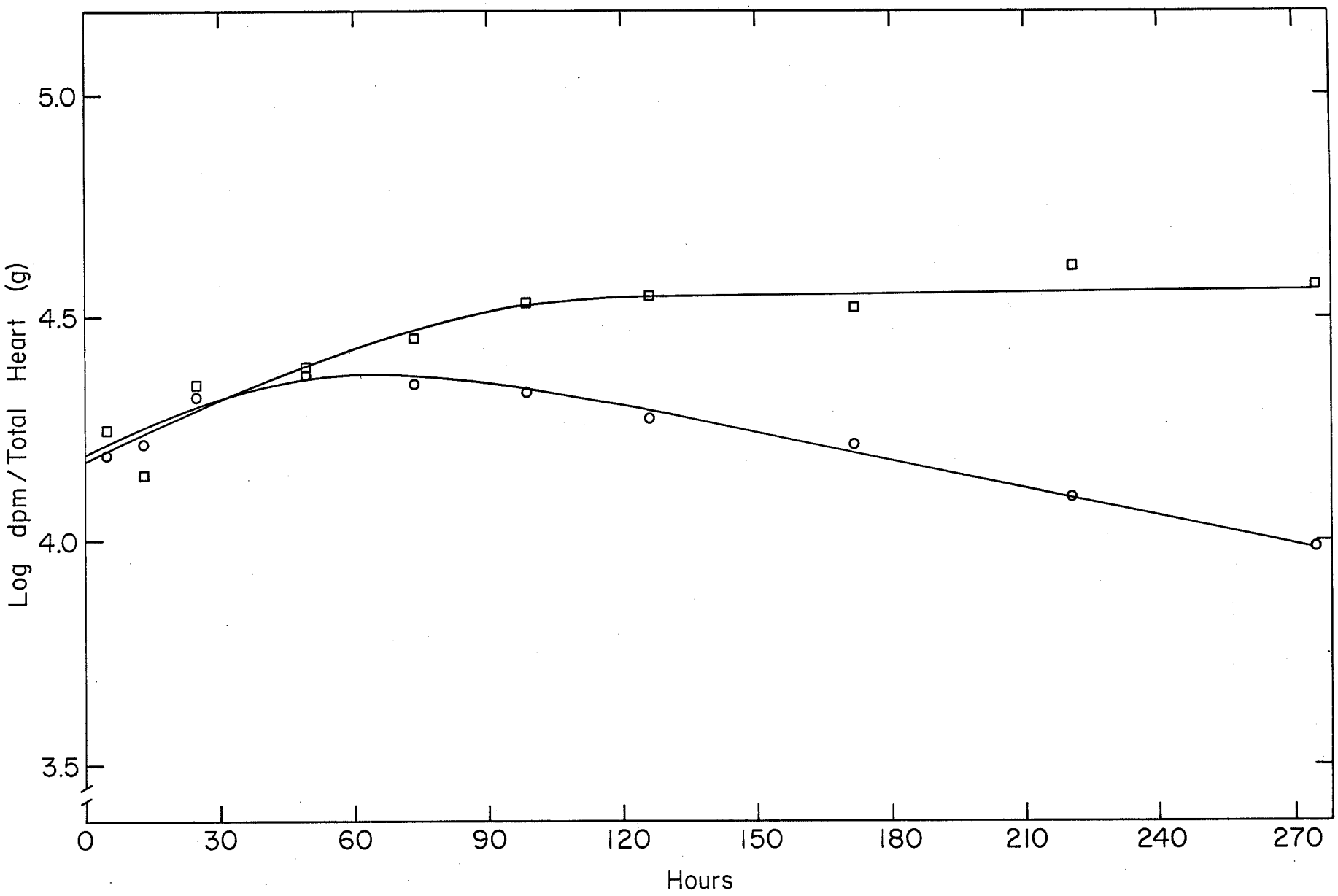
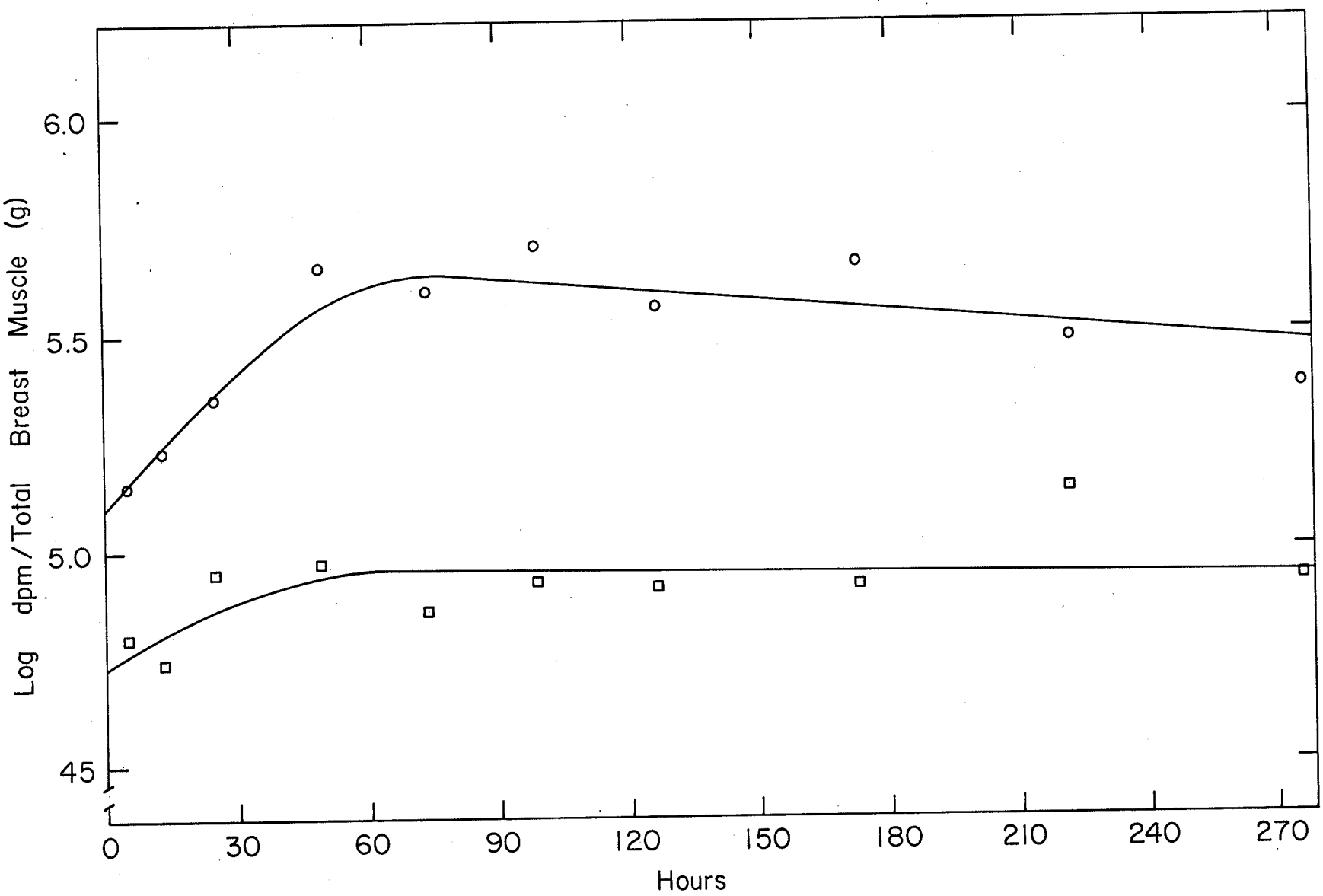

5.0
4.5
4.0
3.5
Log dpm / Total Heart (g)
0
30
60
90
120
150
180
210
240
270
Hours

Figure 18. Plots of Total Radioactivity in Breast Muscle versus Time after Injection of 7-^{14}C-Nicotinic Acid for Quail Fed either the Nicotinic Acid Free Diet (□——□) or Restricted Amounts of the Nicotinic Acid Supplemented Diet (o——o) (Experiment 8).

Each point represents the mean of duplicate observations. Each duplicate consisted of breast muscle samples pooled from two quail each.


6.0
5.5
5.0
45
Log dpm / Total Breast Muscle (g)
0
30
60
90
120
150
180
210
240
270
Hours

The decay curves, however, approached exponential values for liver tissues in both groups during the period 5-13, 25-50, and 74-275 hours after the injection of the isotope. Utilizing this set of time points an estimate of half-lives for the first (5-13 hours), the second (25-50 hours), and the third (74-275 hours) period were 39.9 hours, 221.3 hours and 215.4 hours for the niacin deficient group and 29 hours, 69 hours and 169.6 hours for the restricted group (Table 24). This data would indicate that initially the disappearance of the label in livers of both groups was rapid but was subsequently reduced during the second period and that the much slower decline of label persisted during the third period.

As can be seen in Figures 17 and 18 the patterns of decay of radioactivity in the heart and breast muscle tissues were markedly different from that observed in liver tissues. In breast muscle tissues maximum radioactivity values were observed during the time period of from 20 to 50 hours after which there was no apparent decrease in radioactivity over the duration of the experiment in the niacin deficient group and only a slight decrease in radioactivity in the food-restricted group. These results would suggest that the apparent turnover of these radioactive products in breast muscle tissue of the niacin deficient group approaches zero and the turnover of these products in the food-restricted group was very slow with a half-life of approximately 613 hours.

The overall pattern for heart tissue was similar to that observed for breast muscle tissue except that the half-

Table 24. Calculations of Half-lives of the Radioactive Products in Various Organs After the Injection of 7-^{14}C-Nicotinic Acid. (Experiment 8).[1]

Diet	Half-lives		
	Liver	Heart	Breast Muscle
	hour	hour	hour
NFD (ad libitum)	39.9 (5-13 hr)[2]	-	-
	221.3 (25-50 hr)	-	-
	215.4 (74-275 hr)	infinite	infinite
NFD + 0.007% nicotinic acid (restricted)	29.0 (5-13 hr)	-	-
	69.0 (25-50 hr)	-	-
	169.6 (74-275 hr)	376.6	613.3

[1]Half-lives were calculated from the slope of the least squares regression analysis.

[2]Values in the bracket represent the time intervals chosen for the determination of half-lives of each segment of curves.

Abbreviation: NFD, niacin free diet.

life of these radioactive products in heart tissue was approximately half of that for the food-restricted group whereas in the niacin deficient group there does not appear to be any significant turnover of these products. The results would also suggest that niacin metabolites are highly conserved, particularly under niacin deficiency.

The non-exponential nature of the liver decay curve, a possibly different specific radioactivity (radioactivity/unit of pyridine nucleotides) among tissues, together with the low but gradual uptake of isotope in both the heart and breast muscle tissues may be attributed to a differential rate of exchange of the label among tissues. It would appear that there was a gradual tendency for an equilibrium to be established among the various tissues. The slow uptake in both heart and breast muscle tissues may be partially attributed to the fact that nicotinic acid must be first converted to NAD in the liver followed by catabolism to nicotinamide before it is released into the general circulation (Dietrich, 1971). Nicotinamide would then be in a form that could be utilized by tissues such as heart and breast muscle. The time course for this conversion should not affect overall half-life values as it has been shown that this process is relatively rapid as nearly all nicotinic acid was converted into nicotinic acid, nicotinic acid mononucleotide, and deamido-NAD within 1.0 minute of 7-^{14}C-nicotinic acid administration (Ijichi et al., 1966).

A comparative time course study of the uptake of the two forms of niacin and NAD would establish the relative

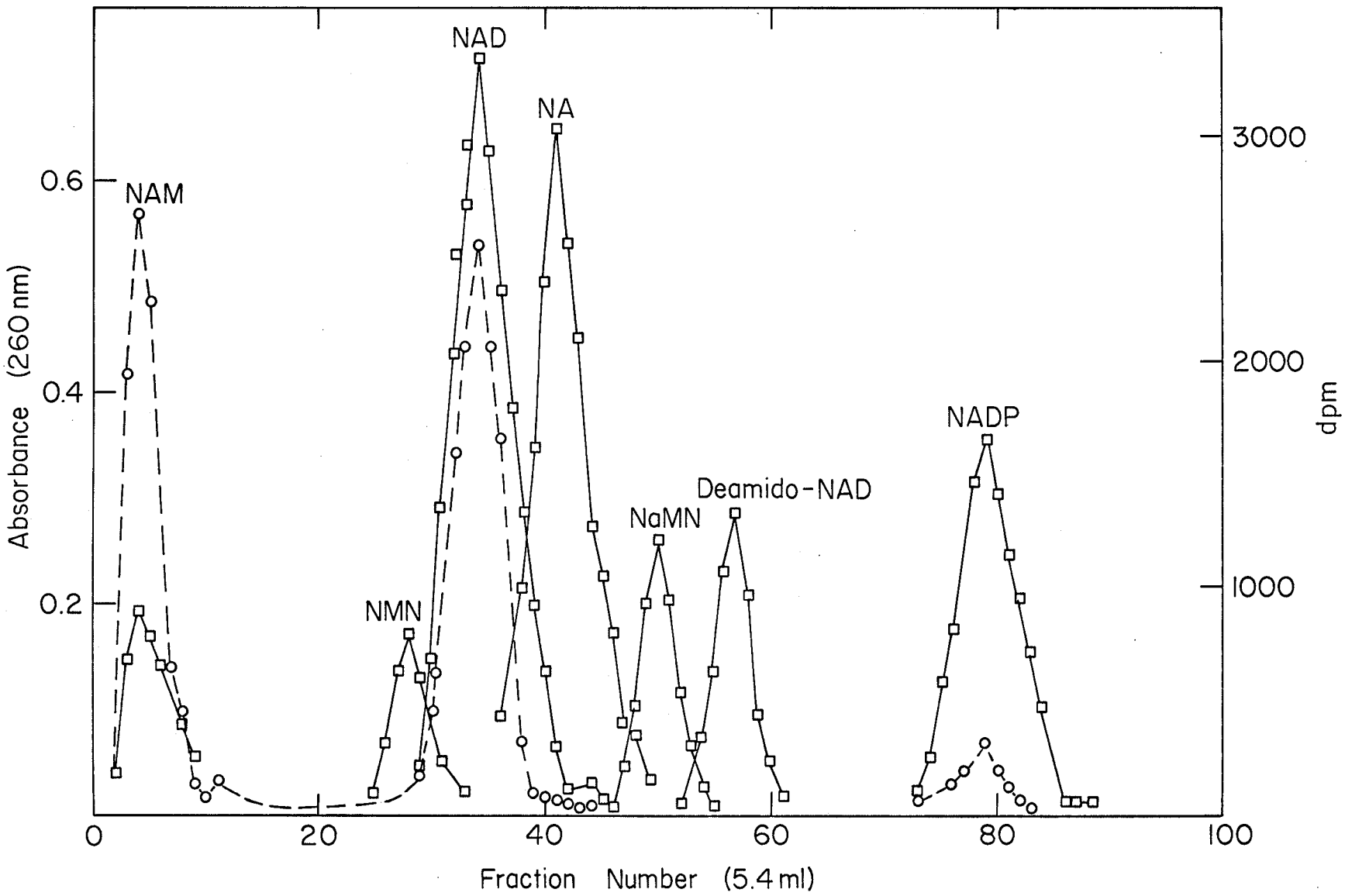
importance of the liver in the metabolism of nicotinic acid, nicotinamide and NAD, the necessity of nicotinic acid to be taken up by the liver before it can be utilized by other tissues and the ability of nicotinamide to be directly utilized by heart and breast muscle tissues. Relatively short term studies (several minutes to a few hours duration) should be carried out to establish the nature of these changes (changes in total and specific radioactivity, radioactivity/unit of pyridine nucleotide) among the different compounds. The long term studies should be repeated using nicotinamide or NAD as the source of isotope if the short term experiment confirms the above hypothesis.

The net effect of the postulated gradual exchange of isotopes among tissues would be to yield apparent turnover rates rather than true turnover rates. The true turnover rates of heart and breast muscle tissues would therefore be shorter than the estimated apparent values. These results nevertheless demonstrate, as indicated above, that pyridine nucleotide turnover in the breast muscle tissue, the major source of total pyridine nucleotide reserves, is low in both groups but almost approaches zero in the niacin deficient group.

A typical DEAE chromatogram for the elution of pyridine nucleotides from a liver extract is illustrated in Figure 19. This procedure provided a basis for establishing the type and proportions of pyridine nucleotides that are formed in liver and breast muscle tissues following the administration of 7-^{14}C-nicotinic acid.

Figure 19. Elution Pattern of Pyridine Nucleotide and Their Derivatives on a DEAE Cellulose Column Chromatography (Experiment 8).

Authentic samples of nicotinamide(2 umoles), nicotinamide mononucleotide(2 umoles), NAD (2 umoles), nicotinic acid(2 umoles), nicotinic acid mononucleotide(2 umoles), deamido-NAD(2 umoles) and NADP(2 umoles) were mixed with 2.3 ml of liver extracts from the food-restricted group and the sample was applied to a DEAE column(1.2 x 20 cm) and eluted as described in Experimental Procedures. The broken lines(O---O) indicate radioactivity and the solid lines(□——□) indicate absorbance at 260 nm. The following abbreviations are used in the Figure: NAM, nicotinamide; NMN, nicotinamide mononucleotide; NAD, nicotinamide adenine dinucleotide; NA, nicotinic acid; NaMN, nicotinic acid mononucleotide; NADP, nicotinamide adenine dinucleotide phosphate.


NAD
NA
NAM
NMN
NaMN
Deamido-NAD
NADP
0.6
0.4
0.2
Absorbance (260 nm)
3000
2000
1000
dpm
0
20
40
60
80
100
Fraction Number (5.4 ml)

The results of this chromatograph demonstrated that nearly all of the radioactivity was associated with only three metabolites, nicotinamide, NAD, and NADP. The other metabolites involved in niacin metabolism are apparently present in negligible concentration including deamido-NAD and nicotinamide mononucleotide.

In this study the radioactive metabolites from two of the time periods were examined: 13 and 221 hours were selected to represent short term and long term patterns for liver (Table 25) or in the case of breast muscle tissue the time periods of 99 and 221 hours were used (Table 26). The initial periods in both cases were selected to represent the time at which maximum accumulation of radioactivity occurred.

The results of this study showed that the major portion of the radioactivity in the liver in both the niacin deficient and food-restricted groups at either time period was associated with nicotinamide (average value 48.0%) and NAD (average value 42.9%). The remainder of radioactivity was associated with NADP (average value 9.2%). The relative percentages of the three radioactive metabolites also were not greatly affected by time of analysis or dietary status. There was however a slight but significant reduction ($P<0.05$) in the percentage of NAD in the food-restricted group in the longer time period (221 hours) as compared to the short time period (13 hours). In addition there was a significant difference ($P<0.05$) between the niacin deficient and food-restricted groups in the percentage of NADP at 221 hours after the injection.

Table 25. Distribution of Radioactive Products in Liver Following DEAE Column Chromatography (Experiment 8).[1]

Radioactive Products	Time After the Injection of 7-^{14}C-nicotinic acid					
	13 hour			221 hour		
N F D (ad libitum)	dpm/g liver	%[2]	dpm/g liver / dpm injected/g BW	dpm/g liver	%[2]	dpm/g liver / dpm injected/g BW
Nicotinamide	353,013±73,781	50.0±8.0	2.71±0.50	107,983±21110	47.6±1.7	0.92±0.16
N A D	296,372±11,994	42.3±3.1	2.28±0.14	100,321±28029	44.0±2.4	0.85±0.22
N A D P	49,792±16,460	7.1±2.0	0.39±0.13	18,475± 5121	8.0±0.3[3c]	0.16±0.04
Others	4,205 ± 310	0.6±0.04	——	682 ± 160	0.3±0.01	——
NFD + 0.007% nicotinic acid (restricted)						
Nicotinamide	204,918±18,251	44.4±0.9	1.73±0.16	17,919 ± 1400	48.7±7.2	0.167±0.00
N A D	210,345± 9,856	46.0±0.9[3a]	1.77±0.09	14,1971± 3150	38.6±2.5[b]	0.134±0.01
NADP	41,379 ±10,845	9.0±1.6	0.35±0.08	4,544 ±1310	12.3±1.7[d]	0.043±0.002
Others	3,210 ±270	0.7±0.04	——	140 ± 10	8.4±0.01	——

[1]Values are mean ± S.E for duplicate observations. Each duplicate consisted of liver samples pooled from 2 birds.

[2]Expressed as % of total radioactivity recovered after chromatography.

[3]Values with different superscripts (a and b) within a row are significantly different ($P<0.05$).
Values with different superscripts (c and d) within a column are significantly different ($P<0.05$).

Abbreviations: NFD, niacin free diet; BW, body weight.

Although the relative proportion of labelled metabolites within a group did not seem to change with time, the relative radioactivity levels $\left\{\frac{\text{dpm/g liver}}{\text{dpm injected/g BW}}\right\}$ changed considerably. In the time period between 13 and 221 hours there was a marked reduction in the relative radioactivity levels in liver of these three labelled metabolites: 66% for nicotinamide, 62.7% for NAD, and 59% for NADP in the niacin deficient group and 90.3% for nicotinamide, 92.4% for NAD, and 87.7% for NADP in the food-restricted group. The results indicate that among the three metabolites, NAD was most severely affected whereas NADP was least affected. These results would also suggest that radioactive metabolites in the food-restricted group were more rapidly replaced as compared to the niacin deficient group.

The distribution of radioactivity in breast muscle among metabolites following DEAE column chromatograph is presented in Table 26. The results show that nearly all of the label was associated with NAD or nicotinamide and none, in contrast with liver, was associated with NADP. Also, within groups there were no significant changes ($P>0.05$) in the percentages of nicotinamide and NAD between the two time periods. There was however a significant reduction (11 versus 25%, $P<0.05$) in the percentage of nicotinamide in the food-restricted group at 99 hours as compared to the niacin deficient group but no significant changes in the percentage of NAD between two groups (89 versus 75%, $P>0.05$). Overall it may be concluded that the proportions of NAD and nicotinamide were not markedly affected by time but there was a trend to reduced proportion

Table 26. Distribution of Radioactive Products in Breast Muscle Following DEAE Column Chromatography (Experiment 8).[1]

Radioactive Products	Time After the Injection of 7-^{14}C-nicotinic acid					
	99 hour			221 hour		
N F D (ad libitum)	dpm/g muscle	%[2]	dpm/g muscle / dpm injected/g BW	dpm/g muscle	%[2]	dpm/g muscle / dpm injected/g BW
Nicotinamide	4,980± 376	25.1±2.6[3a]	0.045±0.00	4,973±1,615	15.8±2.6	0.042±0.01
N A D	14,890±1,439	74.9±5.8	0.129±0.00	26,430±2,599	84.2±4.5	0.23 ±0.01
Others	0	0	0	0	0	0
NFD + 0.007% nicotinic acid (restricted)						
Nicotinamide	8,418 ± 59	10.9±1.0[b]	0.070±0.00	5,411 ±192	11.3±1.0	0.051±0.00
N A D	68,798±9,610	89.1±3.8	0.57 ±0.03	42,439±8,700	88.7±6.7	0.39 ±0.06
Others	0	0	0	0	0	0

[1]Values are mean ± S. E for duplicate observations. Each duplicate consisted of breast muscle samples pooled from 2 birds.

[2]Expressed as % of total radioactivity recovered after chromatography.

[3]Values with different superscripts (a and b) within a column are significantly different ($P<0.05$).

Abbreviations: NFD, niacin free diet; BW, body weight.

of nicotinamide and increased proportion of NAD in the niacin deficient group as compared to the food-restricted group.

The apparent half-lives for individual niacin metabolites were determined from the 13 and 221 hours time points for liver (Table 25) and from the 99 and 221 hours time points for breast muscle (Table 26). The apparent half-lives of nicotinamide, NAD, and NADP in liver tissue in the niacin deficient group were estimated to be 80.3, 100.2, and 626 hours respectively whereas the corresponding values for the food-restricted group were 92.1, 87.7 and 469.2 hours respectively (Table 27).

In breast muscle the apparent half-life of nicotinamide in the restricted group was about 10 times shorter than that of the niacin deficient group (4,300 vs 32,147 hours). Furthermore, the apparent half-life of NAD in the food-restricted group was 490.1 hours whereas the corresponding value was infinite in the niacin deficient group. Interestingly enough, the apparent half-life as determined from the combined nicotinamide and NAD values from each group was nearly the same as the corresponding values presented in Table 24. In this comparison the half-life of NADP was not considered as NADP accounts for only 9.2% of the total radioactivity. These results also indicate that turnover rates of pyridine nucleotides (i.e. NAD and NADP) and their derivative (nicotinamide) vary with the nutritional status and tissues of the animal.

The results (Table 25) in the current study are at variance with the results obtained with rat liver under normal physiological conditions by Gerber and Deroo (1970). These

Table 27. Calculations of Apparent Half-lives of Individual Metabolites in Liver and Breast Muscle Tissues Following the Injection of 7-^{14}C-Nicotinic Acid. (Experiment 8).[1]

Diet		Apparent Half-lives	
		Liver	Breast Muscle
		hour	hour
NFD (ad libitum)			
	Nicotinamide	80.3	32147.3
	N A D	100.2	infinite
	N A D P	626.0	ND[2]
NFD + 0.007% nicotinic acid (restricted)			
	Nicotinamide	92.1	4301.7
	N A D	87.7	490.1
	N A D P	469.2	ND[2]

[1]Half-lives were calculated from the slope of the least squares regression analysis.

[2]Abbreviation: ND, not determined; NFD, niacin free diet.

researchers reported that 70-80% of the radioactivity in various tissues (i.e., liver, muscle and heart) was recovered in NAD and the remaining radioactivity was present in nicotinamide at all times later than 3 hours after the injection of 7-^{14}C-nicotinic acid. These differences may in part have resulted from a difference in dietary status or from a difference in species of animals. In addition, they also reported that no significant radioactivity was recovered in nicotinamide mononucleotide, NADP and deamido-NAD. The percentage of radioactivity associated with muscle tissue NAD was similar in the two experiments.

In the current research with liver, the characteristic metabolic products of nicotinic acid such as nicotinic acid itself or nicotinic acid mononucleotide and deamido-NAD (Preiss and Handler, 1958) were not detectable as reported by Gerber and Deroo (1970). These results would suggest that the levels of these intermediates in the biosynthesis of NAD are very low or that they had been rapidly converted into final products. Previous work with rat livers by Ijichi et al (1966) support these observations. They reported that 7-^{14}C-nicotinic acid appears to be rapidly converted to radioactive NAD by way of nicotinic acid mononucleotide and deamido-NAD. The radioactivity of nicotinic acid, nicotinic acid mononucleotide and deamido-NAD reached the maximum 1.0 minute after the injection. These radioactive intermediates disappeared almost completely within 10 minutes. Radioactivity in NAD, in contrast, increased with time reaching a maximum at about 10 minutes and then declined gradually. Initially the amounts of label

associated with nicotinamide and NADP were also relatively low. The concentration of these compounds gradually increased with time. At 4 hours after the injection, nicotinamide, NAD and NADP were present in the ratio of 3:24:1. Overall the results indicate that nicotinic acid, nicotinic acid mononucleotide and deamido-NAD are utilized very rapidly for the biosynthesis of NAD *in vivo* and therefore are present in the tissue only shortly after being ingested.

Ijichi *et al* (1966) also reported the half-life of ^{14}C-NAD in the liver of rats under normal physiological condition to be about 10 hours. The discrepancies in values between their experiment and the current results may be attributed to a species or nutritional status difference and the time at which the turnover was estimated. These studies would be a reflection of both the rate of degradation of niacin within the liver and the rate at which it was exported to other tissues. The values in the current studies would more accurately reflect liver degradation rate but as indicated above are also affected by the interorgan transfer of radioactivity.

It may be concluded that the incorporation of 7-^{14}C-nicotinic acid in quail tissues is influenced by diet, type of tissue and time after administration of isotope and possibly type of isotope. Overall the turnover rate of radioactive products in various tissues of the niacin deficient group appeared to be much slower relative to that of the food-restricted group. Also the apparent turnover rate of radioactive products in heart and breast muscle in the niacin deficient group was

virtually zero whereas the turnover rate in the food-restricted group was low. The turnover rates of isotope in the liver tissue although low were considerably greater relative to those obtained in the other tissues. This was particularly true for the food-restricted group. The true or actual turnover rates within the different tissues may be somewhat different than apparent values due to a differential degree of uptake and export of label among the various tissues.

Chromatographic separation of the radioactive products demonstrated that most of the radioactivity was associated with nicotinamide, NAD, and NADP in liver and NAD and nicotinamide in breast muscle. The relative turnover rate of these metabolites were also affected by type of tissue and the dietary status of the animal. However, time of sampling after injection of isotope did not have a marked influence on the proportion of isotope. Further research will have to be carried out in order to clarify and establish some of the interacting effects discussed.

PART VI. Effects of Dietary Nicotinic acid Levels on the Metabolism of 7-^{14}C-nicotinic acid in Different Tissues and the Influence of Pyridine Nucleotides on the *in vitro* Thermal Stability of NAD- and NADP-Dependent Enzymes.

(A) Introduction

Part VI a:

The results of the previous study demonstrated that there was a differential rate of niacin metabolism in various organs of the food-restricted and niacin deficient birds. Additional research, however, should be carried out to confirm and extend these observations. The objective of this study was to establish the influence of varying concentrations of dietary nicotinic acid or the nicotinic acid precursor, tryptophan, on the turnover rates of pyridine nucleotides in liver, heart, breast muscle and brain tissues of Japanese quail.

Part VI b:

It is well known that enzyme ligands, particularly coenzymes and substrates, are effective in preventing inactivation of their specific enzymes *in vitro*. This concept has led to the proposal that enzyme-ligand interactions may also be determinants of rates of enzyme degradation *in vivo* (Grisolia, 1964).

Inoue and Pitot (1970) reported that pyridoxal phosphate stabilized crude or partially purified pyridoxal phosphate dependent enzymes against heat denaturation or proteolytic inactivation in vitro. In other studies NAD, the coenzyme of glyceraldehyde-3-phosphate dehydrogenase has been shown to exert profound effects on the structure and function of the enzyme (Marangos and Constantinides, 1974b). This coenzyme has also been shown to be essential for the renaturation of the 8 M urea dissociated glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (Deal and Constantinides, 1967) and yeast (Deal, 1969). Furthermore, Fenselau (1970) demonstrated that NAD is capable of protecting rabbit muscle apoglyceraldehyde-3-phosphate dehydrogenase from limited proteolysis by trypsin and chymotrypsin. The intimate relationship between the coenzyme and enzyme is illustrated by Glock and McLean (1958) on changes in glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase in mammary tissues during lactation. They reported that changes in these enzymes are paralleled by changes in total NADP concentration and in particular, the reduced form. Yonetani and Theorell (1962) also reported that alcohol dehydrogenase was stabilized by the addition of NAD to preincubation mixture.

Another possible role of coenzymes and substrates may be as "directional" agents in promoting the formation of enzymatically active structures. Childson et al. (1966) reported that H-type LDH dissociated with a denaturing agent can be converted into tetrameric form by the addition of NADH which greatly

accelerates the amount of active enzyme. They suggested that the action of the coenzyme is to promote aggregation or to direct the subunits into conformations, which are enzymatically active. Conformational changes induced by a coenzyme were also suggested by the report of Levy et al (1966) on glucose-6-phosphate dehydrogenase. These authors reported that glucose-6-phosphate dehydrogenase of low catalytic activity can be converted to a more active form in the presence of low levels of NADP. They believed that this activation resulted from a conformational change and the role of the coenzymes was to stabilize the functional structures of the enzymes. Overall it may be concluded that the physical properties of different dehydrogenases are affected to a considerable degree by pyridine nucleotides. Little or no information, however, is available with regard to the effects of these compounds on avian enzymes or with regard to comparative stability of several dehydrogenases from different tissues of the niacin deficient animal.

The objective of the second part of this study was to determine if the in vitro stability of liver or breast muscle enzyme from the niacin deficient and food-restricted (but niacin supplemented) quail was affected by the concentration of added pyridine nucleotides in incubation mixture. Information obtained in this study should provide a basis for estimating the influence of niacin status on turnover rates of various metabolically related enzymes.

(B) Experimental Procedures

EXPERIMENT 9

(i) Management of Quail

Newly hatched quail were provided a mixed diet consisting of the niacin free diet and commercial chick starter diet until they reached approximately 24 g. The diet adapted quail were randomized, weighed and separated into five groups from 20 to 25 each. Each group of quail was placed on one of the following powdered diets: (i) group I received the niacin free diet plus 0.007% nicotinic acid; (ii) group II received the niacin free diet; (iii) group III received the group I diet in amounts sufficient to maintain their body weights at the same levels as those of the niacin deficient quail (group II); (iv) group IV received the niacin free diet plus 0.66% tryptophan and (v) group V received the niacin free diet plus 0.07% nicotinic acid. All birds were fed these diets ad libitum except for group III which received the diet once or twice a day.

After 7 days on their respective diets the quail on the niacin free diet developed niacin deficiency symptoms characterized by poor growth and feather development. Prior to the injection of the isotope on day 7, all quail were fasted for 7 hours. Groups II and III received 2.0 μCi (per 31 g body weight) of 7-^{14}C-nicotinic acid (specific activity of 61 mCi/mmol) whereas groups I, IV and V received 2.5 μCi (per 45.5 g body weight) of 7-^{14}C-nicotinic acid through intraperitoneal administration. After injection, four quail from each group

were sacrificed at various time intervals: 71 hours, 147 hours, 217 hours and 286 hours. These time intervals, on the basis of the previous experiment, were considered to be optimal.

Throughout the experiment all quail were housed in electrically heated stainless steel batteries with wire floors. The weights of quail were measured daily. At the time of sacrifice the brain, heart, liver and breast muscle were promptly removed, immediately plunged into liquid nitrogen, weighed and stored at $-70^{o}C$ until required for analysis. The levels of pyridine nucleotides were determined as described in the General Experimental Procedures. Orthogonal polynomial contrasts were used to examine the trends of curves of radioactive products following 7-^{14}C-nicotinic acid injection (Snedecor and Cochran, 1967). Half-lives were calculated using least squares regression analysis (Dixon and Brown, 1977) from the equation $t_{\frac{1}{2}} = 0.693/k$, where k is the slope of the first order activity decay curve.

(ii) Preparation of Radioactive Samples

An adequate amount of frozen brain, heart, liver and breast muscle from quail was weighed and homogenized in cold 0.6 N $HClO_4$ for 1.0 minute with a Polytron homogenizer (Gerber and Deroo, 1970). Homogenates were pooled in proportion to tissue weights and then centrifuged at 12,000 x g for 10 minutes in a Sorvall RC 2B centrifuge. After centrifugation, 0.1 or 0.2 ml aliquots of the protein-free supernatant were transferred to scintillation vials for counting. The determination of radioactivity was the same as described in the previous experiment.

(iii) Preparation of Enzyme Extracts Employed in the Thermal Inactivation Studies were as follows:

a) Incubation of enzyme extracts in the absence of pyridine nucleotides

Liver and breast muscle tissues of quail were homogenized in an appropriate volume (5 ml) of 10 mM potassium phosphate buffer (pH 7.5) containing 5 mM 2-mercaptoethanol and 2 mM EDTA for 1.0 minute with a Polytron homogenizer and were centrifuged at 50,000 x g for 15 minutes in a Sorvall RC 2B centrifuge. Aliquots of each preparation were incubated in an insulated water bath for 15 minutes at several temperatures between 27°C and 65°C. Heating was performed in small glass tubes (inside diameter 4 mm) which were closed by rubber membranes so as to minimize evaporative changes which would cause an increase in enzyme concentration and a reduction in temperature. After heating, the enzyme solutions were immediately placed in an ice water bath with initial mixing. After 5 minutes the samples were centrifuged at 50,000 x g for 15 minutes in order to remove the denatured proteins and then assayed. Enzyme activity measurements were as outlined in General Experimental Procedures.

b) Incubation of enzyme extracts in the presence of pyridine nucleotides

The procedures for the preparation and incubation of the enzyme extracts were identical to those described in section (a) except that the reaction mixtures for incubation contained NAD or NADP. In this experiment an appropriate volume of 10 mM NAD

or 5 mM NADP was added to the reaction mixtures prior to incubation at different temperatures. The final concentrations of NAD or NADP in the incubation mixtures were 1 mM or 0.5 mM respectively. Enzyme activities are expressed as a percent of the initial activity in a buffered extract just prior to incubation.

(C) Results and Discussion

1. Effect of Nicotinic acid Deficiency on the Growth and Content of Pyridine Nucleotides in Various Organs.

The results of this study demonstrate that there were no significant differences in body, brain, heart, liver and breast muscle weights among groups I, IV and V at all indicated time intervals but that these three groups were significantly ($P<0.01$) different from groups II and III in all parameters except for the 71 and 147 hour brain weights (Table 28). There were also no significant differences ($P>0.05$) in body, brain, heart, liver and breast muscle weights between groups II and III. The significant threeway interaction ($P<0.01$, Table 29) among diets, tissues and time by a factorial analysis suggests that there is a complex interrelationship among these factors.

As reported previously the niacin deficient quail not only failed to gain body weight but also showed typical niacin deficiency symptoms. These symptoms were not observed in the food-restricted quail even though the average body weight of this group was approximately the same as the niacin deficient quail. It was also of interest to note that growth was not

Table 28. Effects of 5 Different Diets on Body, Brain, Heart, Liver and Breast Muscle Weight of Quail (Experiment 9)[1,2].

Group/Diet		71 hours after 7-^{14}C-nicotinic acid injection				
		Body Wt	Brain Wt	Heart Wt	Liver Wt	Breast muscle Wt
		g	g	g	g	g
I	NFD + 0.007% nicotinic acid (ad libitum)	57.9±2.5^{A}	0.60±0.02^{A}	0.53±0.02^{a}	1.96±0.14^{a}	8.98±0.53^{A}
II	N F D (ad libitum)	35.5±1.7^{B}	0.52±0.01^{B}	0.39±0.02^{b}	1.36±0.12^{b}	4.14±0.48^{B}
III	NFD + 0.007% nicotinic acid (restricted)	36.8±1.0^{B}	0.55±0.01^{AB}	0.28±0.01^{b}	1.22±0.05^{b}	4.94±0.24^{B}
IV	NFD + 0.66% tryptophan (ad libitum)	64.3±5.5^{A}	0.59±0.01^{A}	0.52±0.05^{a}	2.07±0.11^{a}	10.34±1.37^{A}
V	NFD + 0.07% nicotinic acid (ad libitum)	56.4±2.6^{A}	0.56±0.01^{AB}	0.47±0.04^{a}	2.32±0.45^{a}	8.49±0.90^{A}

Group	147 hours after 7-^{14}C-nicotinic acid injection				
	Body Wt	Brain Wt	Heart Wt	Liver Wt	Breast muscle Wt
	g	g	g	g	g
I	67.5±2.2^{A}	0.61±0.01^{a}	0.47±0.02^{A}	2.06±0.15^{A}	11.42±0.60^{A}
II	38.0±1.1^{B}	0.54±0.01^{a}	0.31±0.02^{B}	1.09±0.07^{B}	5.32±0.30^{B}
III	35.5±1.9^{B}	0.55±0.02^{a}	0.29±0.01^{B}	1.30±0.13^{B}	4.52±0.54^{B}
IV	62.3±6.5^{A}	0.58±0.03^{a}	0.47±0.04^{A}	1.94±0.06^{A}	10.35±0.48^{A}
V	60.6±1.8^{A}	0.59±0.01^{a}	0.50±0.01^{A}	2.08±0.17^{A}	9.58±0.34^{A}

Continued

[1]Values are mean ± S.E of 4 birds.

[2]Means not sharing a common superscript letter within a column are significantly different ($P<0.01$ or $P<0.05$).

Abbreviations: NFD, niacin free diet; wt, weight.

Table 28. Continued.

Group	Body Wt	Brain Wt	Heart Wt	Liver Wt	Breast muscle Wt
	217 hours after 7-^{14}C-nicotinic acid injection				
	g	g	g	g	g
I	76.1±2.9^{A}	0.65± 0^{A}	0.61±0.3^{A}	2.05±0.10^{A}	12.92±0.87^{A}
II	34.7±4.1^{B}	0.52±0.02^{B}	0.28±0.04^{B}	1.04±0.17^{B}	3.91±0.80^{B}
III	35.1±4.2^{B}	0.55±0.01^{B}	0.24±0.02^{B}	1.00±0.13^{B}	4.45±0.80^{B}
IV	77.8±2.0^{A}	0.63± 0^{A}	0.62±0.03^{A}	2.25±0.09^{A}	13.26±0.48^{A}
V	75.9±2.9^{A}	0.64±0.02^{A}	0.56±0.02^{A}	2.41±0.31^{A}	12.79±0.47^{A}
	286 hours after 7-^{14}C-nicotinic acid injection				
Group	Body Wt	Brain Wt	Heart Wt	Liver Wt	Breast muscle Wt
	g	g	g	g	g
I	85.4±5.6^{A}	0.66±0.01^{A}	0.79±0.05^{A}	2.51±0.35^{A}	16.69±2.35^{A}
II	32.2±2.6^{B}	0.52±0.01^{B}	0.26±0.02^{B}	0.92±0.15^{B}	3.27±0.89^{B}
III	33.2±3.3^{B}	0.56±0.01^{B}	0.24±0.02^{B}	0.92±0.15^{B}	3.65±0.83^{B}
IV	88.1±3.8^{A}	0.66±0.01^{A}	0.68±0.05^{A}	2.56±0.17^{A}	16.61±0.70^{A}
V	86.7±5.6^{A}	0.63±0.01^{A}	0.65±0.07^{A}	2.42±0.13^{A}	17.26±1.36^{A}

Table 29. Analysis of Variance of Data in Table 28 (Experiment 9).

Source of Variation	df	Sum of Square	Mean Square	F
Diet	4	463.2275	115.8069	115.22**
Tissue	3	4329.6406	1443.2134	1435.85**
Time	3	56.5012	18.8337	18.74**
Diet x Tissue	12	891.62221	74.3018	73.92**
Diet x Time	12	77.6749	6.4729	6.44**
Tissue x Time	9	144.0964	16.0107	15.93**
Diet x Tissue x Time	36	171.9640	4.7768	4.75**
Error	256	257.3132	1.0051	
Total	335	6356.4414		

**Significant at $P<0.01$.

Abbreviation: df, degree of freedom.

stimulated when the dietary concentration of nicotinic acid was increased by 10 fold (0.07%) or when the level of tryptophan was increased by 3 fold (0.66%).

The results from Table 30 show that there were no significant differences ($P>0.05$) in levels of NAD in the brain, heart, and liver of quail among the five different groups whereas the level of NAD in the breast muscle of the niacin deficient group was significantly ($P<0.01$) lower than that of the other four groups. It also demonstrates that the addition of nicotinic acid and tryptophan in excessive amounts to the niacin free diet did not alter the levels of NAD in any of the tissues relative to the control group (I). These results are in agreement with those reported previously (Part III, Experiment 6).

2. Effects of Dietary Nicotinic acid Levels on the Metabolism of 7-^{14}C-nicotinic acid in Various Tissues.

A comparison of the relative radioactivity levels and the type of decay curves of radioactive products in various tissues is presented in Tables 31-34. The decay curves for liver, heart, breast muscle and brain tissues for all groups except the niacin deficient group generally demonstrated linear functions ($P<0.01$). The exception in the former group was breast muscle tissue from the food-restricted group. The decay curve of this tissue which was not significant ($P>0.05$) consisted of 68% linearity and 29% cubicity. This may in part be due to errors in experimental analysis. In contrast to all

Table 30. Effects of 5 Different Diets on the Levels of NAD in Brain, Heart, Liver and Breast Muscle of Quail (Experiment 9). [1,2]

Group/Diet		Brain	Heart	Liver	Breast Muscle
		µmoles/g brain	µmoles/g heart	µmoles/g liver	µmoles/g muscle
I	NFD + 0.007% nicotinic acid (ad libitum)	0.37±0.02[a]	0.48±0.03[a]	0.63±0.02[a]	0.99±0.02[B]
II	NFD (ad libitum)	0.36±0.03[a]	0.45±0.03[a]	0.59±0.02[a]	0.57±0.02[A]
III	NFD + 0.007% nicotinic acid (restricted)	0.37±0.03[a]	0.46±0.02[a]	0.58±0.02[a]	1.03±0.03[B]
IV	NFD + 0.66% tryptophan (ad libitum)	0.40±0.04[a]	0.47±0.04[a]	0.64±0.06[a]	1.06±0.03[B]
V	NFD + 0.07% nicotinic acid (ad libitum)	0.40±0.04[a]	0.50±0.05[a]	0.66±0.06[a]	1.14±0.04[B]

[1]Values are mean ± S.E for duplicate observations. Each duplicate consisted of tissue samples pooled from two quail.

[2]Means not sharing a common superscript letter within a column are significantly different ($P<0.01$).

Abbreviation: NFD, niacin free diet.

Table 31. Incorporation of 7-^{14}C-Nicotinic Acid into Brain Tissue of Quail of Various Time Intervals (Experiment 9).[1]

Group/Diet		Activity [(dpm/g of brain)/(dpm injected/g of body weight)]				
		71 hour	147 hour	217 hour	286 hour	Type of decay curve
I	NFD + 0.007% nicotinic acid (ad libitum)	0.40±0.00	0.41±0.01	0.38±0.00	0.27±0.00	linear**
II	NFD (ad libitum)	0.40±0.04	0.32±0.02	0.38±0.03	0.37±0.00	see footnote
III	NFD + 0.007% nicotinic acid (restricted)	0.44±0.00	0.39±0.04	0.28±0.04	0.19±0.02	linear**
IV	NFD + 0.66% tryptophan (ad libitum)	0.44±0.02	0.42±0.04	0.48±0.30	0.32±0.02	linear**
V	NFD + 0.07% nicotinic acid (ad libitum)	0.24±0.02	0.16±0.00	0.09±0.00	0.05±0.01	linear**

[1]Values are mean ± S.E for duplicate observations. Each duplicate consisted of brain samples pooled from 2 birds.

[2]Type of decay curve for NFD (Group II) was determined by orthogonal polynomial contrast. The NFD decay curve consisted of 57% quadratic and 43% cubic function ($P>0.05$).

**: significant ($P<0.01$).

Abbreviation: NFD, niacin free diet.

Table 32. Incorporation of 7-^{14}C-Nicotinic Acid into Heart Tissue of Quail at Various Time Intervals (Experiment 9).[1]

Group/Diet		Activity [(dpm/g of heart)/(dpm injected/g of body weight)]				
		71 hour	147 hour	217 hour	286 hour	Type of[2] decay curve
I	NFD + 0.007% nicotinic acid (ad libitum)	0.72±0.09	0.64±0.03	0.37±0.05	0.29±0.00	linear**
II	NFD (ad libitum)	0.59±0.05	0.83±0.05	0.87±0.06	0.85±0.01	see footnote
III	NFD + 0.007% nicotinic acid (restricted)	0.80±0.03	0.59±0.01	0.47±0.00	0.29±0.00	linear**
IV	NFD + 0.66% tryptophan (ad libitum)	0.83±0.03	0.50±0.00	0.52±0.06	0.32±0.05	linear**
V	NFD + 0.07% nicotinic acid (ad libitum)	0.35±0.05	0.26±0.00	0.16±0.01	0.08±0.01	linear**

[1]Values are mean ± S.E for duplicate observations. Each duplicate consisted of heart samples pooled from 2 birds.

[2]Type of decay curve for NFD (Group II) was determined by orthogonal polynomial contrast. The NFD decay curve consisted of 46% linear and 43% quadratic function ($P>0.05$). **: significant at $P<0.01$.

Abbreviation: NFD, niacin free diet.

Table 33. Incorporation of 7-^{14}C-Nicotinic Acid into Liver Tissue of Quail at Various Time Intervals (Experiment 9)[1]

Group/Diet		Activity [(dpm/g of liver)/(dpm injected/g of body weight)]				
		71 hour	147 hour	217 hour	286 hour	Type of decay curve[2]
I	NFD + 0.007% nicotinic acid (ad libitum)	1.45±0.04	0.58±0.01	0.40±0.03	0.25±0.02	linear**
II	NFD (ad libitum)	3.85±0.14	3.95±0.27	1.77±0.03	1.77±0.33	linear**
III	NFD + 0.007% nicotinic acid (restricted)	1.58±0.17	0.64±0.01	0.37±0.03	0.28±0.00	linear**
IV	NFD + 0.66% tryptophan (ad libitum)	0.75±0.03	0.27±0.02	0.26±0.02	0.14±0.01	linear**
V	NFD + 0.07% nicotinic acid (ad libitum)	0.35±0.06	0.17±0.00	0.07±0.01	0.05±0.00	linear**

[1] Values are mean ± S.E for duplicate observations. Each duplicate consisted of liver samples pooled from 2 birds.

[2] **: significant at $P<0.01$.

Abbreviation: NFD, niacin free diet.

Table 34. Incorporation of 7-^{14}C-Nicotinic Acid into Breast Muscle Tissue of Quail at Various Time Intervals. (Experiment 9).

Group/Diet		Activity [(dpm/g of breast muscle)/(dpm injected/g of body weight)]				
		71 hour	147 hour	217 hour	286 hour	Type of[2] decay curve
I	NFD + 0.007% nicotinic acid (ad libitum)	0.51±0.01	0.64±0.07	0.66±0.01	0.56±0.06	linear**
II	NFD (ad libitum)	0.17±0.04	0.20±0.04	0.27±0.05	0.25±0.05	see footnote
III	NFD + 0.007% nicotinic acid (restricted)	0.50±0.14	0.41±0.03	0.52±0.05	0.43±0.08	see footnote
IV	NFD + 0.66% tryptophan (ad libitum)	0.53±0.07	0.63±0.05	0.73±0.02	0.57±0.05	linear*
V	NFD + 0.07% nicotinic acid (ad libitum)	0.38±0.02	0.39±0.05	0.31±0.00	0.25±0.05	linear

[1]Values are mean ± S.E for duplicate observations. Each duplicate consisted of breast muscle samples pooled from 2 birds.

[2]Type of decay curve for NFD and food-restricted groups was determined by orthogonal polynomial contrast. The decay curve for quail fed diet II (NFD) consisted of 49% linear and 51% quadratic function ($P>0.05$). For the restricted group(III) it was 68% linear and 29% cubic function ($P>0.05$). *: significant at $P<0.05$; **: significant at $P<0.01$. Abbreviation: NFD, niacin free diet.

other treatment groups the decay pattern of the niacin deficient group was quadratic ($P>0.05$) with the exception of liver tissue (i.e. linear). The quadratic function in heart and breast muscle tissues may be attributed to a progressive increase in label for 217 hours after injection followed by a slight decrease at 286 hours.

The significant threeway interaction ($P<0.01$, Table 35) among diets, tissues, and time after injection in the amount of radioactivity incorporated into tissues suggests that there is a complex interrelationships among these factors. The data from the Tables 31-34 reveal that there is a considerable variation in the relative radioactivity levels of radioactive products in different tissues. The initial relative radioactivity after the injection was highest in liver, followed by heart, breast muscle and brain in that order for all groups irrespective of the nature of diets. The average respective 71 hour values for the different tissues were 1.6 for liver, 0.66 for heart, 0.42 for breast muscle and 0.38 for brain. This indicates a considerable variation in the initial uptake of isotope among tissues. Similar observations were also made among treatment groups. In the case of liver, the initial relative radioactivity levels of the niacin deficient group (II) was the highest while that in the high niacin-fed group (V) was the lowest. This may be attributed to the competition during absorption between the labelled and unlabelled nicotinic acid. The nicotinic acid and tryptophan supplemented groups (I, III and IV) tended to have intermediate values as compared to groups II and V. Breast

Table 35. Analysis of Variance of Data in Tables 31-34 (Experiment 9).

Source of Variation	df	Sum of Square	Mean Square	F
Diet	4	11.9073	2.9768	449.87**
Tissue	3	8.9040	2.9680	448.53**
Time	3	3.4487	1.1496	173.72**
Diet x Tissue	12	27.9059	2.3255	351.44**
Diet x Time	12	1.0055	0.0838	12.66**
Tissue x Time	9	5.0528	0.5614	84.84**
Diet x Tissue x Time	36	5.1973	0.1444	21.82**
Error	80	0.5294	0.0066	
Total	159	63.9510		

**Significant at $P<0.01$.

Abbreviation: df, degree of freedom.

muscle tissue in contrast showed a different pattern. The uptake of radioactivity in this tissue was lowest in quail fed the niacin free diet as compared to those fed the other diets whereas the opposite pattern occurred in liver tissue. The uptake of radioactivity in heart tissue followed more or less the same trends as in the breast muscle except that the uptake of radioactivity in the niacin deficient group was not the lowest. With regard to brain tissue, all treatments were similar except for the high nicotinic acid fed quail (group V) which had a much lower level of radioactivity incorporated than the other groups. The pattern also changed with time due to a differential rate of turnover of nicotinic acid in various tissues and among quail fed the different diets.

The results from Table 31-34 which are summarized in Table 36 would suggest that the turnover rates (the reciprocal of half-life) of radioactive nicotinic acid metabolites in all tissues of the niacin deficient quail (group II) were extremely low whereas the turnover rates were much higher in quail (group V) fed the diet that contained an excessively high level of nicotinic acid. The turnover rates of the quail fed the high tryptophan but zero nicotinic acid containing diet were intermediate to the niacin deficient quail (group II) and those fed the optimum nicotinic acid containing diets (groups I and III). The turnover rates of nicotinic acid metabolites for all tissues except the brain tissue were also similar for quail that were fed the same diet under restricted (group III) or ad libitum conditions (group I).

Table 36. Calculations of Half-lives of the Radioactive Products in Various Organs after the Injection of 7-^{14}C-Nicotinic Acid (Experiment 9).[1]

Group/Diet	Half-lives (hour)			
	Brain	Heart	Liver	Breast Muscle
I NFD + 0.007% nicotinic acid (ad libitum)	491.5	385.0	189.3	infinite
II NFD (ad libitum)	infinite	infinite	276.1	infinite
III NFD + 0.007% nicotinic acid (restricted)	366.7	355.4	179.5	infinite
IV NFD + 0.66% tryptophan (ad libitum)	infinite	430.4	210.6	infinite
V NFD + 0.07% nicotinic acid (ad libitum)	203.8	218.6	148.7	infinite

[1]Half-lives were calculated from data presented in Tables 31, 32, 33 and 34 using the least squares repression analysis.

Abbreviation: NFD, niacin free diet.

With regard to specific tissues the pattern was also very different. Nicotinic acid metabolites in breast muscle tissue appeared to have an extremely low rate of turnover while the rate in liver tissue, although low, was much higher than that in breast muscle. Heart and brain tissues had values that were intermediates to those of liver and breast muscle tissues.

Overall the results of this study are in agreement with the results from the previous studies and would suggest as discussed previously, that there is a considerable influence of diet and tissue on nicotinic acid turnover rate. The results of this study would also suggest that the half-life values of nicotinic acid metabolites are affected by a differential rate of niacin exchange among tissues. This was discussed in the previous section. The results of this study also confirms the previous observations that the level of pyridine nucleotides (NAD in the current study) are markedly depressed in breast muscle tissue but not in other tissues in the niacin deficient quail. It can be speculated that the level of niacin in essential organs under conditions of extremely severe niacin deficiency may also become depleted. The animal would presumably die as indicated in a previous experiment (Experiment 4) when this situation developed. These results also support the previous observation that the best overall index of the niacin status in quail is the level of pyridine nucleotides in the muscle tissue.

3. Thermal Stability Studies

Thermal stability studies were carried out on selected liver and breast muscle enzymes from three experimental groups (Table 37 and 38). The corresponding activity levels of malic enzyme from liver tissue for groups I, II, and III when incubated at 50°C for 15 minutes were 18%, 21% and 16% of initial activities respectively ($P>0.05$). Liver glyceraldehyde-3-phosphate dehydrogenase from the control group (I) retained 53 or 7% of its initial activity when incubated at 50°C or 55°C for 15 minutes, respectively. The enzymes from the niacin deficient and food-restricted groups in contrast retained 19 or 2% and 31 or 3% of their respective initial activity under the same conditions. However, there were no significant differences ($P>0.05$) in the relative degree of inactivities among the three groups. Liver lactic dehydrogenase from the control group (I), the niacin deficient group (II) and the food-restricted group (III) retained 87, 93 and 81% respectively of their initial activities when incubated at 55°C for 15 minutes ($P>0.05$). A similar pattern was also observed at 60°C. The percent activity loss of aldolase from all three groups appeared to follow a similar pattern to that of lactic dehydrogenase. These results would suggest that *in vitro* there was not a large difference among the three treatment groups in the thermal stability of liver enzymes. Liver pyridine nucleotide patterns, as indicated previously, were also not affected by the niacin status of the diet.

Table 37. Studies on Thermal Inactivation of Liver Enzymes (Experiment 9). [1,2]

Incubation Temperature (°C)	Malic enzyme						Glyceraldehyde-3-phosphate dehydrogenase					
	(1) Control		(2) N F D		(3) Restricted		(1) Control		(2) N F D		(3) Restricted	
	units/g liver	%[3]	units/g liver	%	units/g liver	%	units/g liver	%	units/g liver	%	units/g liver	%
27°C	46.7±1.6	100	25.7±17.2	100	41.4±14.4	100	180.9± 3.9	100	156.1±22.0	100	151.0±19.3	100
50°C	8.4±3.6	17.7±7.0[a]	4.5± 2.4	20.8±4.8[a]	7.3± 3.9	16.3±3.7[a]	96.1±13.3	53.0±6.2[a]	28.6± 0.2	18.8±2.4[a]	49.8±27.2	31.0±14.0[a]
55°C	0	0	0	0	0	0	11.8± 3.5	6.6±2.0[a]	3.5± 0.7	2.3±0.1[a]	4.3± 3.0	2.6±1.7[a]
60°C	0	0	0	0	0	0	0	0	0	0	0	0
	Lactic dehydrogenase						Aldolase					
	(1) Control		(2) N F D		(3) Restricted		(1) Control		(2) N F D		(3) Restricted	
	units/g liver	%	units/g liver	%	units/g liver	%	units/g liver	%	units/g liver	%	units/g liver	%
27°C	566.1±2.1	100	334.8±22.3	100	439.5±38.7	100	171.1±17.0	100	102.7±8.1	100	130.5±22.2	100
50°C	—	—	—	—	—	—	—	—	—	—	—	—
55°C	489.6±49.3	86.5±8.4[a]	310.7±19.0	92.8±0.3[a]	349.8±17.8	80.6±11.1[a]	138.9±13.0	81.2±0.4[a]	85.2±6.5	83.0±0.2[a]	102.6±12.2	79.3±4.1[a]
60°C	296.5±13.4	52.4±2.1[a]	179.8± 2.7	54.0±4.4[a]	212.2±13.8	49.0± 7.4[a]	89.4±10.2	52.2±0.8[a]	49.7±7.8	48.1±3.8[a]	61.6±6.7	47.7±3.0[a]

[1]Values are mean ± S.E for quadruple observations. Each quadruple represents pooled liver samples from 2 quail.
[2]Means in the same row not sharing a common superscript letter are significantly different ($P<0.05$).
[3]Percent activities are expressed as the percentage of their respective initial activities at 27°C.
Abbreviations: NFD, niacin free diet group; restricted, food-restricted group.

In breast muscle the percent (23%) of the initial activity of glyceraldehyde-3-phosphate dehydrogenase that was recovered from the niacin deficient group (II) was significantly ($P<0.01$) less than that from both the control group (52%) and the food-restricted group (45%) when enzyme extract was incubated at 50°C for 15 minutes (Table 38). There were, however, no significant differences between the control and food-restricted groups ($P>0.01$). At 50°C, breast muscle LDH from the control group (I) retained 87%, the niacin deficient group 81% and the food-restricted group 86% of their initial activities respectively ($P>0.05$). Under the same conditions breast muscle aldolase from the control group retained 90%, the niacin deficient group 92% and the food-restricted group 91% of their initial activities respectively ($P>0.05$). These results would suggest that dietary nicotinic acid levels affected the thermal stability of glyceraldehyde-3-phosphate dehydrogenase but not LDH or aldolase.

Other researchers have also studied the influence of vitamin deficiency and tissue cofactor levels on the stability of certain enzymes. Litwack and Rosenfield (1973) showed that the rates of *in vivo* degradation of several pyridoxal enzymes correlate well with the *in vitro* dissociation of the enzyme into a apoenzyme and pyridoxal phosphate. Recently Lee *et al* (1977) indicated that the cofactor dissociation may influence the enzyme stability, and to some extent the differences in degree of cofactor saturation were correlated with differences of enzyme stability, whether animals were vitamin B_6

Table 38. Studies on Thermal Inactivation of Breast Muscle Enzyme (Experiment 9).[1,2]

Incubation Temperature (°C)	Glyceraldehyde-3-phosphate dehydrogenase						Lactic dehydrogenase					
	(1) Control		(2) N F D		(3) Restricted		(1) Control		(2) N F D		(3) Restricted	
	units/g muscle	%[3]	units/g muscle	%	units/g muscle	%	units/g muscle	%	units/g muscle	%	units/g muscle	%
27°C	342.3±10.4	100	259.7±29.4	100	276.4±5.6	100	1576.7±273.2	100	1063.9±194.5	100	1222.4±97.1	100
50°C	175.8± 5.1	51.7±2.9[A]	57.5± 3.7	22.5±1.6[B]	124.9±9.8	45.3±2.6[A]	1348.8±190.1	86.8±6.0[a]	850.3±139.1	81.1±5.4[a]	1052.4±120.3	86.0±5.7[a]

	Aldolase					
	(1) Control		(2) N F D		(3) Restricted	
	units/g muscle	%	units/g muscle	%	units/g muscle	%
27°C	329.8±30.9	100	250.1±61.2	100	232.3±17.0	100
50°C	296.9±36.2	89.5±3.2[a]	220.3±39.0	91.7±7.9[a]	211.0±15.0	91.0±0.9[a]

[1] Values are mean ± S. E for quadruple observations. Each quadruple represents pooled breast muscle samples from 2 birds.

[2] Means in the same row not sharing a common superscript letter are significantly different ($P<0.01$).

[3] % Activities are expressed as the percentage of their respective initial activities at 27°C.

Abbreviations: NFD, niacin free diet group; restricted, food-restricted group.

deficient or not. They postulated that coenzyme binding and rate of degradation, although not causally related, are both reflections of structural properties of the enzymes which determine coenzyme binding and degradation rate. Garcia-Bunuel et al (1962) reported that the depletion of nicotinic acid in a diet caused a marked lowering of pyridine nucleotide levels in rat liver and activities of liver isocitrate dehydrogenase or glycerophosphate dehydrogenase in niacin deficient rats were 25 or 50% less relative to those of the control rats. Katunuma et al (1971) reported that niacin deficiency resulted in the appearance of an intestinal enzyme which would inactivate apolactic and apoglutamic dehydrogenases. This would have the effect of increasing the synthesis of NAD- or NADP-specific proteases.

The results from the current study demonstrate that the degree of inactivation of pyridine nucleotide dependent enzymes as well as aldolase in liver tissue at all indicated incubation temperatures was not affected by the nature of the diet which is consistent with the observation that pyridine nucleotide levels in liver tissue were also not affected by the nature of the diet.

With regard to muscle tissue a different pattern was obtained. The greater sensitivity of glyceraldehyde-3-phosphate dehydrogenase to heat denaturation in the niacin deficient group as compared to the other groups would suggest a causative relationship between enzyme stability and NAD status of the tissue. It may be hypothesized that the low level of NAD in

the breast muscle tissue may have been directly responsible for the decreased enzyme stability or its deficiency may have indirectly caused the accumulation of certain tissue metabolites or proteases which in turn had a destabilizing effect on the enzyme. As discussed in Part III, the affinity of NAD for glyceraldehyde-3-phosphate dehydrogenase is much stronger than that for LDH. If the dissociation of NAD from enzyme plays a crucial role in enzyme degradation, LDH should be more readily destabilized relative to glyceraldehyde-3-phosphate dehydrogenase due to its high dissociation constant. However, the observation from the current study showed an opposite result. Jaenicke (1969) reported that rabbit muscle apoglyceraldehyde-3-phosphate dehydrogenase was more labile than apolactic dehydrogenase. Therefore, the failure of niacin deficiency to effect the stability of LDH may be attributed to the intrinsic properties of its structure.

4. Effect of Pyridine Coenzymes on Heat Inactivation

The results from Figures 20 and 21 demonstrated that all of the liver and breast muscle enzymes were protected by NAD or NADP against thermal inactivation. The relative activities of liver enzymes in the presence and absence of added NAD or NADP were: malic enzyme 68 and 16% at 50^{o}C, glyceraldehyde-3-phosphate dehydrogenase 88 and 17% at 55^{o}C, LDH 90 and 51% at 60^{o}C and aldolase 88 and 50% at 60^{o}C. The corresponding activities of breast muscle enzymes were: glyceraldehyde-3-phosphate dehydrogenase 100 and 0% at 55^{o}C,

Figure 20. Thermal Inactivation of Quail Liver Enzymes (Experiment 9).

The enzyme solutions were incubated for 15 minutes at the indicated temperatures. The final concentrations of NAD or NADP in the incubation mixtures were 1mM or 0.5 mM , respectively. Enzyme activities were expressed as a percent of the initial activity in a buffered extract just prior to incubation.

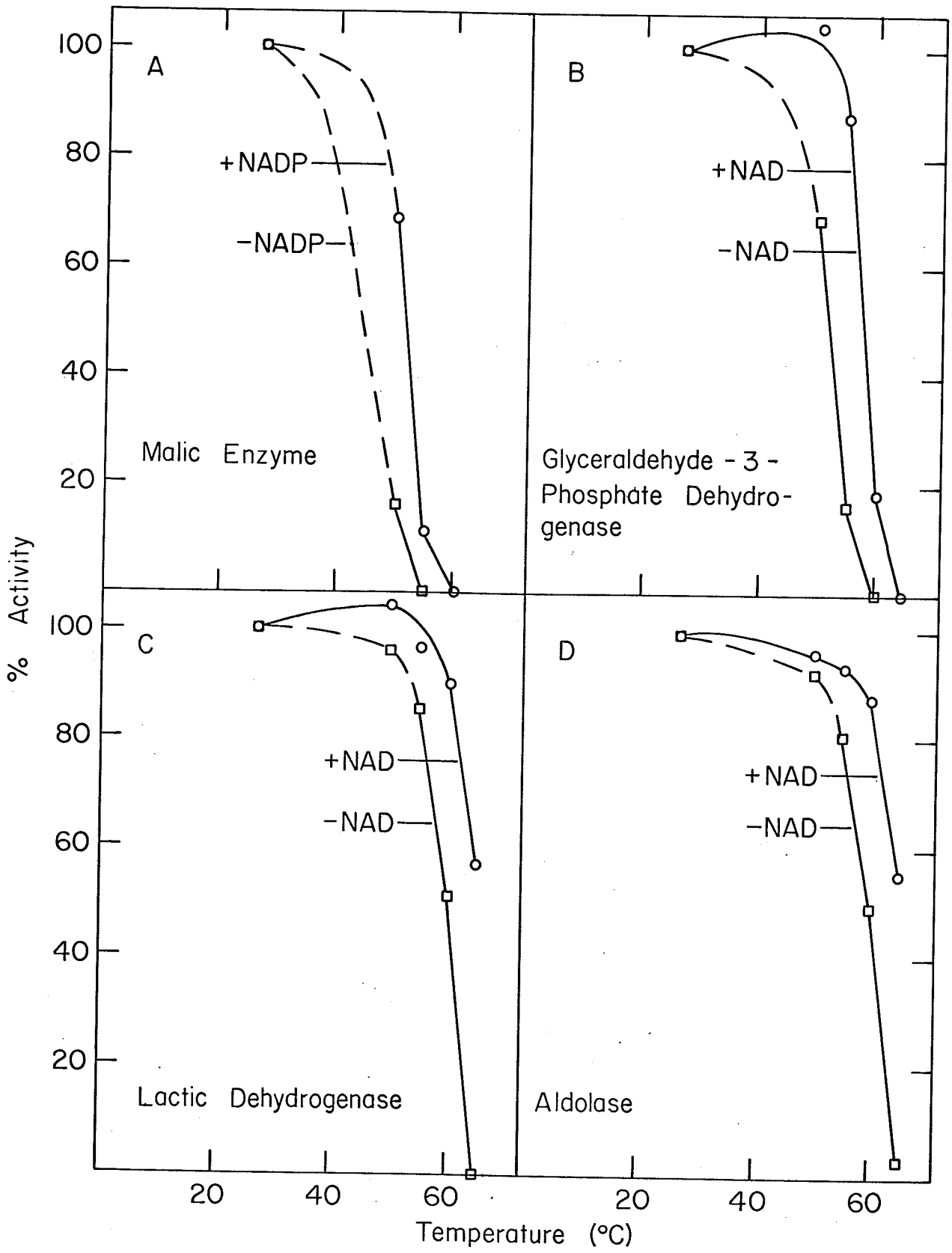
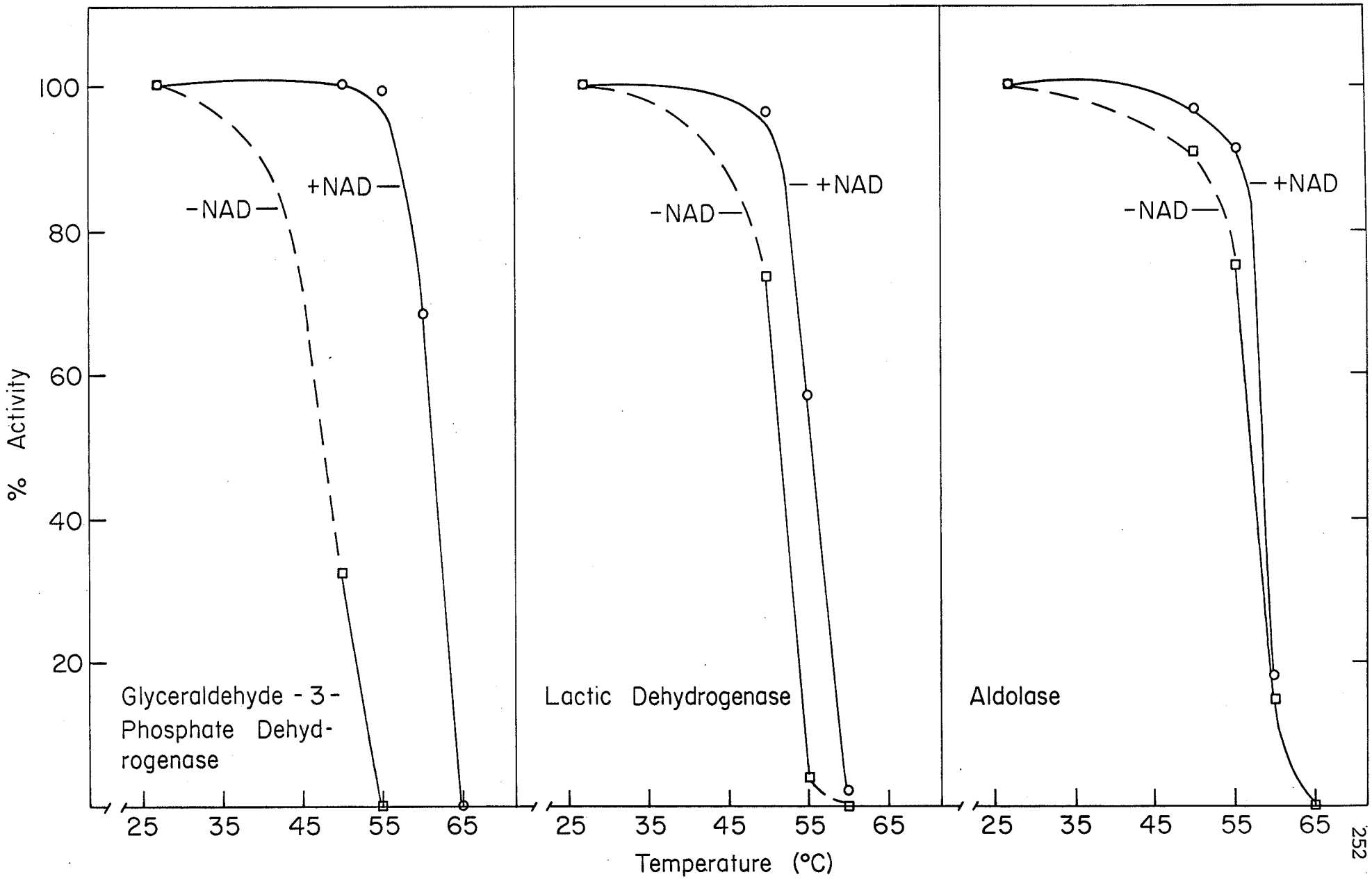

A
+NADP
-NADP
Malic Enzyme
B
+NAD
-NAD
Glyceraldehyde - 3 - Phosphate Dehydro-genase
C
+NAD
-NAD
Lactic Dehydrogenase
D
+NAD
-NAD
Aldolase
% Activity
100
80
60
40
20
20
40
60
Temperature (°C)

Figure 21. Thermal Inactivation of Quail Breast Muscle Enzymes (Experiment 9).

The enzyme solutions were incubated for 15 minutes at the indicated temperatures. The final concentration of NAD in the incubation mixtures was 1 mM. Enzyme activities were expressed as a percent of the initial activity in a buffered extract just prior to incubation.


100
80
60
40
20
% Activity
-NAD
+NAD
Glyceraldehyde - 3 - Phosphate Dehyd- rogenase
Lactic Dehydrogenase
Aldolase
25
35
45
55
65
Temperature (°C)

LDH 57 and 4% at 55°C and aldolase 91 and 75% at 55°C. Malic enzyme was not assayed for in the breast muscle tissue as it has been shown to be absent in this tissue (Utter, 1959). These results would indicate that in the absence of coenzymes, liver malic enzyme and glyceraldehyde-3-phosphate dehydrogenase were slightly more sensitive to heat treatment than LDH and aldolase. In the breast muscle glyceraldehyde-3-phosphate dehydrogenase appeared to be the most sensitive of all enzymes to thermal inactivation in the absence of NAD whereas in the presence of NAD the pattern was completely reversed. In contrast, LDH and particularly aldolase were protected against thermal inactivation by NAD to a much lesser degree. Overall these results would suggest that in the absence of coenzymes the pyridine nucleotide dependent enzymes in the breast muscle tissue appeared to be more unstable to heat treatment than the corresponding enzymes in liver tissue.

The difference in response between liver and breast muscle tissues may be attributed to the type and concentration of enzymes and/or relative concentration of coenzymes to enzymes and other metabolites that may affect the stability of the enzymes. A correlation of structural differences between multiple forms of the same enzyme and their individuality of turnover might also be anticipated. It has been well established that the predominant form of aldolase isozyme in the liver tissue is aldolase B suited for fructose-1,6-diphosphate synthesis (gluconeogensis) whereas in the muscle only aldolase A is present which is responsible for cleavage of

fructose-1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate and thus favors glycolysis (Rutter et al, 1968). These enzymes have different physicochemical and enzymatic properties. It is also known that LDH isozymes differ markedly from each other with respect to their physicochemical properties and tremendous differences in turnover exist for individual isozymes between different tissues. With respect to the possibility that thermal denaturations play a role in LDH degradation, Zondag (1963) and Vesell and Yielding (1966) reported that LDH-1(B_4) in certain animals is more heat stable than LDH-5(A_4). The former is degraded 22 times faster than the latter in rat skeletal muscle, although LDH-5 cardiac muscle is degraded almost 8 times faster than cardiac muscle LDH-1 (Fritz et al, 1970). Muscle glyceraldehyde-3-phosphate dehydrogenase exhibits a negative cooperativity in coenzyme binding whereas liver enzyme has a positive co-operativity in coenzyme binding (Conway and Koshland, 1968; Kirschner et al, 1971). This discrepancy, in the case of liver enzyme, may reflect its function in gluconeogenesis. Other differences between glyceraldehyde-3-phosphate dehydrogenase from muscle and liver tissues were also seen in the number of sulfhydryl groups, NAD binding, allosteric properties and regulatory effects exerted by inorganic phosphate (Heinz and Kulbe, 1971). Variation among tissues in enzyme stability may therefore be a reflection of the properties of the different isozymes. A second factor that may account for part of the difference is that the level of metabolites surrounding enzymes and the

localization of enzymes in cells. These factors presumably would not only influence *in vitro* but also *in vivo* enzyme stabilities.

The mode by which NAD protects dehydrogenases against thermal inactivation and the relationship between thermal stability and degradation rate was not established in the current study although a considerable amount of related information is available from the literature with regard to the effect of pyridine nucleotide on properties and structures of dehydrogenases. Some time ago, Grisolia (1964) pointed out that ligands can protect enzymes against proteolytic attack and suggested that the conformation of an enzyme may determine its susceptibility to extracellular proteases. Citri (1973) has compiled data to demonstrate that many ligands that influence susceptibility to proteolysis cause conformational changes in enzymes. Bond (1975) reported that the addition of NAD to extracts fully protected glyceraldehyde-3-phosphate dehydrogenase from inactivation by all of the proteases tested and partially protected glycerophosphate and lactic dehydrogenases. In addition, it was shown that the long-lived enzymes are generally resistant to proteases and heat while the short-lived enzymes are generally inactivated partially or completely by proteases and heat, with the exception of tyrosine aminotransferase.

There is evidence in the literature however which indicates that thermal instability is not an important factor in initiating the degradation of intracellular proteins. For

instance, Kuehl and Sumison (1970) have found that the rates of degradation of three glycolytic enzymes *in vivo* are similar, while their thermal stabilities *in vitro* are quite different.

In the case of flounder muscle glyceraldehyde-3-phosphate dehydrogenase, the enzyme has been shown to exist as a mixture of forms with each containing a distinct amount of bound NAD (Marangos and Constantinides, 1974b). Therefore, it was inferred that the bound NAD on each form stabilizes it by causing the molecule to assume a more compact configuration that is less susceptible to attack by various inactivation agents such as proteases. The fact that NAD has a further stabilizing effect on the holoenzymes suggests that the added NAD which presumably binds loosely to the enzymes is able to further alter the conformation (Fenselau , 1970). Constantinides and Deal (1969) indicated that NAD stabilizes the quarternary structure of glyceraldehyde-3-phosphate dehydrogenase. They also proposed the possibility that NAD controls the structural and functional heterogeneity of the enzyme as well as maintaining and protecting it against inactivation.

The results of our study support the proposal that added NAD produces an additional stabilizing effect on glyceraldehyde-3-phosphate dehydrogenase and that this may occur because of the refolding of the loosely bound subunits of this enzyme. The refolding mechanism of glyceraldehyde-3-phosphate dehydrogenase also is different from the one

observed for non-cooperative tetramers like LDH (Jaenicke, 1974; Rudolph et al, 1977) or aldolase (Engelhard et al, 1976).

The results of the current study demonstrate that the activities of several dehydrogenases and aldolase are variably protected in vitro against thermal inactivation by either NAD or NADP. It was also shown that there are distinct differences in the pattern of thermal stability of dehydrogenases depending on the source of tissues or levels of coenzymes. It may be hypothesized that the rate of degradation of certain muscle dehydrogenases such as glyceraldehyde-3-phosphate dehydrogenase may be increased in the niacin deficient animals as certain of these enzymes appear to be highly unstable in the presence of very low concentration of pyridine nucleotides. It is conceivable that a decrease in coenzyme brought about by niacin deficiency may result in a diminution in the level of apoenzyme which may be readily denatured in vivo. Liver enzymes, in contrast, are probably not affected by niacin status since pyridine nucleotide levels are not decreased under conditions of niacin deficiency. Pyridine nucleotides also do not seem to provide selective protection of only dehydrogenases as aldolase (a non pyridine nucleotide requiring enzyme) was also partially protected by these coenzymes. Nevertheless, additional research should be carried out to establish the in vitro pattern of response to thermal inactivation of other dehydrogenases and related enzymes in the breast muscle tissue of niacin deficient quail. Also an assay of metabolic inter-

mediates in the tissues of normal and deficient quail would assist in identifying crossover points at which reactions become rate limiting. Information such as this would provide a basis for predicting effects of tissue coenzymes on enzyme stabilities and for identifying rate limiting steps. Follow up studies would be to establish the effects of niacin deficiency on protein turnover.

PART VII : Effect of Niacin Deficiency on General Protein Turnover in Various Tissues of Japanese Quail

(A) Introduction

The protein content of mammalian tissues is usually determined by the balance between rates of protein synthesis and degradation (Goldberg and Dice, 1974). In general, the larger subunits of proteins in eukaryote tissues have a tendency to be degraded more rapidly than smaller ones (Schimke et al., 1973). Dice and Goldberg (1975a) found a correlation between the logarithm of the subunit molecular weight and the half-life of 33 rat liver proteins. They also reported that a highly significant correlation was observed between rates of degradation and isoelectric points for 22 proteins from rat liver (1975b).

Ideally protein turnover studies should be carried out in mature animals that are in a steady state condition (Arias et al., 1969). It is not possible, however, to utilize an ideal condition when studying the influence of niacin deficiency on protein turnover as niacin deficiency could not be induced in the mature quail and even if it was, conditions would probably deviate from steady state conditions because of the niacin deficient state.

In the current study, immature quail were employed and the non-steady state conditions were partially corrected for by maintaining both the niacin deficient and food-

restricted quail in a non-growth state prior to and during the experiment. Currently no literature is available on the influence of niacin status on protein turnover.

The results of the previous study, however, would suggest that the rates of synthesis and degradation of proteins are altered in the niacin deficient animal. This is supported by the observation that the breast muscle tissue underwent a severe atrophy in the niacin deficient animal, and that the stability of certain enzymes was decreased in the breast muscle tissue of the niacin deficient animal.

The objective of the study was to determine if niacin deficiency altered the rate of protein turnover in various tissues of immature quail. The double isotope technique of Arias et al. (1969) will be employed to establish the effects of niacin deficiency on total protein turnover rates and on rates of turnover of proteins of different molecular size and isoelectric charge.

(B) Experimental Procedures

EXPERIMENT 10 :

(i) Management of Animal

Newly-hatched quail were maintained on a mixture of the niacin free diet and the commercial chick starter diet until they reached approximately 21 g. This procedure enabled the quail to adapt to the synthetic diet. Then they were wingbanded, weighed and divided into three groups of similar mean body weights of from 12 to 16 quail each and

were fed the following diets: (i) group I (control), niacin free diet plus 0.007% nicotinic acid; (ii) group II, niacin free diet; (iii) group III, the same diet as group I.

All diets were fed ad libitum except for group III, which received amounts of diets just sufficient to maintain their body weights the same as those of group II. Pair-fed group III was offered the control diet once or twice a day. Quail were housed in electrically heated stainless steel batteries with wire floors and were offered their respective diets in powder form. Weights of quail were measured daily and symptoms of niacin deficiency were carefully checked throughout the course of the experiment. Quail growth in the niacin free group (II) was markedly reduced within a 7 day period which on the basis of the previous experiments indicated that the quail were in a state of severe niacin deficiency. The niacin deficient quail along with the quail from the other two groups were administered radioactive isotopes after being on the test diet for 7 days.

(ii) Double-Isotope Administration

The double-isotope technique described by Arias et al. (1969) was used to determine the relative turnover rates of proteins. All quail from the 3 groups were fasted for 5 hours prior to the injection of the isotope. Initially 8 quail from each group were given an intraperitoneal injection of 9.5 uCi of U-^{14}C-leucine (specific activity of 348 mCi/mmol) in a volume of 0.5 ml and food was restored 3 hours

later. Six days after the initial injection the fasting regimen was repeated, but the quail were injected with 68 uCi of L-[4,5-^{3}H] leucine (specific activity of 119 Ci/mmol) in a volume of 0.5 ml and were killed 4 hours later. In a separate control experiment,4 quail from each group received 4.9 uCi L-U-^{14}C-leucine and 35 uCi L-[4,5-^{3}H]-leucine simultaneously and were sacrificed 4 hours later. At sacrifice the brain, heart, liver, and breast muscle were promptly excised and placed into liquid nitrogen and organs were weighed to the nearest 0.01 g. All data were compared using analysis of variance and significant differences between means were identified by the Newman-Keuls test (Kirk, 1968).

(iii) Preparation of Total TCA-precipitable Proteins from Tissues

Precipitation and washing of proteins were performed according to the procedure of Siekevitz (1952). In each treatment group tissue samples (0.1 - 0.3 g) from 8 quail were individually prepared by slicing the frozen tissues with a scalpel. Each sample was homogenized in 5.0 ml of buffer (20 mM potassium phosphate, 5 mM EDTA, 2 mM 2-mercaptoethanol; pH 7.5) with a Polytron homogenizer (Kinematica, GMbH) at maximum speed for 1.0 minute at 0^{o}. Equal volume of cold 10% TCA was added to the homogenate to precipitate protein. After centrifugation at 50,000 x g for 10 minutes the pellet was washed thoroughly with cold 5%

TCA and recentrifuged as before. This washing was repeated once. The precipitate was suspended in 15 ml of a 1 : 1 mixture of petroleum ether and ethanol, allowed to extract for 30 minutes at room temperature and centrifuged at 50,000 x g for 10 minutes to remove the lipid components. The precipitate was resuspended in 15 ml of ether and centrifuged at 50,000 x g for 10 minutes to remove TCA. After ether extraction the protein pellets were dried in an oven at 45°C.

The dry protein samples were wetted in 0.2 ml of distilled water prior to the addition of 1.0 ml NCS tissue solubilizer. After protein samples were solubilized at 45°C for 15 hours the samples were neutralized with 0.04 ml of glacial acetic acid and suspended in 10 ml of scintillation fluid (16.6 g of 2,4-diphenyloxazole, 0.3 g of 1,4-bis (2-(5-phenyloxazolyl)) benzene and 1 ℓ of Triton X-100 in 2 ℓ of toluene). Samples were allowed to stand at 4°C in the dark for at least 24 hours to reduce chemiluminescence and were then counted using external standardization.

(iv) Fractionation of Breast Muscle Supernatant Proteins on Sephadex G-200

The procedure of Dehlinger and Schimke (1970) was followed. Adequate portions of breast muscle of each treatment group were homogenized in 4 volumes of an equimolar mixture of 50 mM Tris-glycine (pH 8.9) containing 20 mM 2-mercaptoethanol with a Polytron homogenizer for 30 seconds. Four homogenate samples from each group were

pooled on an equal weight basis. The homogenate was brought to 1% SDS so as to dissociate the proteins into subunits by the addition of 1/10 volume of 10% SDS. After 1 hour at room temperature the homogenate was centrifuged at 40,000 x g in a Sorvell RC-2B for 10 minutes. The supernatant was extensively dialyzed against large volumes of an equimolar mixture of 50 mM Tris-glycine (pH 8.9) containing 20 mM 2-mercaptoethanol and 0.1% SDS at room temperature. After centrifugation at 40,000 x g for 5 minutes, 4.0 ml samples, containing approximately 100 - 200 mg of protein, were applied to a Sephadex G-200 column (2.5 x 70 m) equilibrated in the above buffer at room temperature. The column flow rate was 10 ml/hour, and 3.5 - 4.0 ml fractions were collected. Protein samples were precipitated with 1.0 ml of 50% TCA and washed once with 2 ml of 5% TCA. After centrifugation at 40,000 x g for 10 minutes, the protein precipitate was extracted three times with excessive amounts of ether to remove TCA. The protein samples were solubilized in 0.2 ml of distilled water and 1.0 ml of NCS tissue solubilizer at 45^{o}C for 15 hours. Then the protein samples were neutralized with 0.04 ml of glacial acetic acid prior to the addition of 10 ml of scintillation fluid and counted using a Nuclear Chicago Mark II scintillation spectrometer. Results were corrected for quenching by external standards and expressed as dpm.

The control experiment was carried out in duplicate using four animals per group. The column was calibrated in

a separate run with marker proteins prepared in the same fashion as the breast muscle supernatant proteins in the test experiment. Ovalbumin, conalbumin, and cytochrome C were detected by monitoring optical density at 280 nm.

(v) Isoelectric Focusing of Breast Muscle Proteins in Polyacrylamide Gels

a) Preparation of protein samples for isoelectric focusing

The procedure of Klose (1975) was followed with certain modifications. Appropriate portions of breast muscle of each treatment group were homogenized in 10 volumes of distilled water with a Polytron homogenizer at the maximum speed for 30 seconds. Four homogenate samples from each group were pooled on an equal weight basis in each separate experiment. Adequate volume of the disrupting solution, containing 10% Triton X-100, 10 mM borate, 5 mM EDTA and 1 mM PMSF (phenylmethylsulfonyl fluoride, an inhibitor of proteolytic enzymes), was added to the pooled samples to give a final buffer concentration that was one-fifth of the disrupting solution. The sample solutions were allowed to solubilize at room temperature for 30 minutes and centrifuged at 40,000 x g for 10 minutes. The collected supernatants were extensively dialyzed against 4 ℓ of 1% Triton X-100, 2 mM borate, and 1 mM EDTA for 3 hours with two changes of solution.

b) Isoelectric focusing in polyacrylamide gels

Isoelectric focusing in polyacrylamide gels with pH 3.0 - 10 Ampholines (LKB Instruments, Inc.) was performed according to the procedures of Klose (1975) with modifications as outlined below. Isoelectric focusing was carried out using a disc electrophoresis unit at room temperature.

The routine procedure used gels that were 0.6 cm in diameter and 16 cm in length; the total volume of such a gel was 4.32 ml. The gel mixture consisted of 2.0 ml of 5% acrylamide, 0.17% bisacrylamide, 4.6% of sucrose (Aristar), and 1% of Ampholine (0.6% of pH 5 - 8 and 0.4% of pH 3.0 - 10, Ampholine). The pH 5 - 8 Ampholine was added to the mixture as most of the breast muscle proteins tended to focus within this range. To this was added 2.3 ml of a mixture of protein sample (approximately 100 mg) containing 0.04 ml of disrupting solution. After all reagents were thoroughly mixed, 0.02 ml of ammonium persulfate (100 mg/ml) was added, the solution was mixed, degassed quickly by vacuum, and transferred to glass tubes (0.6 x 16 cm) for polymerization at room temperature for at least 2 hours.

Each isoelectric focusing run contained one gel for the determination of pH gradient and one marker gel that contained cytochrome C. The upper (anode) compartment of the apparatus was filled with 5% ($^{V}/_{V}$) phosphoric acid, the lower (cathode) compartment with 5% ($^{V}/_{V}$) ethylenediamine. The apparatus, equipped with 12 tubes, was connected to a Gelman Instrument Power Supply unit and the power was regu-

lated as follows: 1/2 hour, 50 V; 1 hour, 100 V; 16 hours, 150 V. At the end of the run the power was raised to 300 V for 30 minutes.

The gel that was used for the determination of the pH gradient was cut in 2 mm of cross sections with a Gilson aliquogel fractionator (Gilson Medical Electronics). Each slice was extracted with 0.5 ml of distilled water, vortexed and allowed to stand at least for 1 hour at room temperature before the pH was measured. The radioactive sample gels were fixed in 5% TCA for at least 1 hour to precipitate the protein and to bleach out the Ampholines. Then the gels were rinsed with the distilled water several times in order to remove TCA prior to staining. Protein bands were visualized by staining gels in a solution of 0.2% bromphenol blue (acetic acid : H_2O : ethanol, 1 : 9 : 10 $^{V}/_{V}$) for 2½ hours at room temperature. Destaining was performed by washing the gels in a solution (acetic acid : H_2O : ethanol, 1 : 13 : 6 $^{V}/_{V}$). After the termination of destaining, the gels were photographed and scanned at 585 nm in a Gilford model gel scanner linked to a Gilford model 2400 spectrophotometer.

For the determination of radioactivity, the gels which were previously destained were sliced manually and dissolved in 0.5 ml of 30% H_2O_2 at 45^{o}C overnight. One ml of NCS and 0.04 ml of glacial acetic acid were added to the dissolved gels. Finally, 10 ml of toluene-based scintillation fluid were added to each vial. Sample vials

prepared in this way were kept at 4°C in the dark for at least 1 day prior to counting with Nuclear Chicago Mark II scintillation spectrometer. Each sample was counted twice for sufficient time to insure accurate counting. Results were corrected for quenching by external standards and expressed as dpm.

(C) Results and Discussion

A summary of the effect of niacin deficiency and food restriction on body, brain, heart, liver and breast muscle weights is given in Table 39. Control quail gained 39.3 g at the end of the experiment whereas the niacin deficient and food-restricted quail gained 10 and 9.8 g, respectively ($p < 0.01$, between former and latter two groups). Similarly, average brain ($p < 0.05$), heart ($p < 0.01$), liver ($p < 0.01$), and breast muscle ($p < 0.01$) weights of the control group were significantly higher than those of the niacin deficient and food-restricted groups. The results presented in Table 39 are in agreement with the data obtained from the previous experiment (Experiment 9). Niacin deficiency symptoms were also similar to those observed in the previous experiments.

Table 40 summarizes a control experiment in which quail were given U-^{14}C-leucine and [4,5-^{3}H]-leucine simultaneously. A similar $^{3}H/^{14}C$ ratio among all diets suggests that both isotopes were being metabolized in a similar manner in each tissue and, therefore, can be used as companion

Table 39. Effects of Niacin Deficiency and Food Restriction on Body, Brain, Heart, Liver and Breast Muscle Weights of Quail (Experiment 10). [1,2]

Diet	Initial BW	Final BW	Wt. Gain	Brain Wt.	Heart Wt.	Liver Wt.	Breast Muscle Wt
	g	g		g	g	g	g
1) NFD + 0.007% nicotinic acid (ad libitum)	20.9±0.9[a]	60.2±2.4[A]	39.3±1.0[A]	0.60±0.01[a]	0.47±0.03[A]	1.75±0.07[A]	9.42±0.53[A]
2) NFD (ad libitum)	23.2±1.0[a]	33.2±0.4[B]	10.0±0.6[B]	0.56±0.01[b]	0.28±0.01[B]	0.92±0.06[B]	3.65±0.30[B]
3) NFD + 0.007% nicotinic acid (restricted)	22.8±1.1[a]	32.6±1.3[B]	9.8±0.9[B]	0.54±0.01[b]	0.27±0.01[B]	0.84±0.05[B]	3.91±0.27[B]

[1]Values are mean ± S. E of 8 observations.

[2]Means not sharing a common superscript letter within a column are significantly different ($P<0.05$ or $P<0.01$).

Abbreviations: NFD, niacin free diet; BW, body weight; wt, weight.

Table 40. $^{3}H/^{14}C$ Ratios of Total Proteins from Quail Tissues with Simultaneous Injection of Isotopes (Experiment 10).[1,2]

Diet	Brain			Heart		
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
	dpm x 10^{-5}/total brain			dpm x 10^{-5}/total heart		
1) NFD + 0.007% nicotinic acid (ad libitum)	1.32±0.13[a]	0.61±0.05[a]	2.17±0.02[a]	1.57±0.11[A]	0.70±0.05[A]	2.24±0.01[a]
2) N F D (ad libitum)	1.82±0.37[a]	0.83±0.16[a]	2.18±0.01[a]	1.93±0.32[A]	0.88±0.15[A]	2.21±0.02[a]
3) NFD + 0.007% nicotinic acid (restricted)	1.10±0.21[a]	0.55±0.09[a]	1.99±0.08[a]	0.70±0.15[B]	0.34±0.06[B]	2.06±0.03[a]

[1]Values are mean ± S.E of 8 observations. Four quails from each treatment received a simultaneous intraperitoneal injection of 4.9 µCi of U-^{14}C-leucine and 35 µCi of [4,5-^{3}H]-leucine. Four hours later the animals were killed, and the total proteins were prepared and counted.

[2]Means not sharing a common superscript letter within a column were significantly different ($P<0.05$ or $P<0.01$).

Abbreviation: NFD, niacin free diet.

Table 40. Continued.

Diet	Liver			Breast muscle		
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
	dpm x 10^{-5}/total liver			dpm x 10^{-5}/total breast muscle		
1) NFD + 0.007% nicotinic acid (ad libitum)	4.07±0.22^{A}	1.60±0.08^{A}	2.54±0.20^{a}	16.08±1.14^{A}	9.60±0.06^{A}	1.68±0.11^{a}
2) N F D (ad libitum)	4.07±0.21^{A}	1.64±0.18^{A}	2.48±0.15^{a}	8.24±1.2^{B}	4.91±0.99^{B}	1.68±0.07^{a}
3) NFD + 0.007% nicotinic acid (restricted)	2.65±0.27^{B}	0.92±0.09^{B}	2.88±0.02^{a}	3.33±0.23^{C}	1.85±0.06^{C}	1.79±0.06^{a}

isotopes in dual isotope measurements.

The $^{3}H/^{14}C$ ratios in Table 41 reveal that liver had the fastest turnover rates of proteins (the highest $^{3}H/^{14}C$ ratio) and heart and brain had intermediate rates whereas the breast muscle had the slowest turnover rates of proteins (the lowest $^{3}H/^{14}C$ ratio). These observations are in accord with Don and Masters' (1976) reports that considerable variations in $^{3}H/^{14}C$ ratios are noticeable in the total proteins of various tissues, with the relative turnover rate being fastest in reproductive tissues, liver, spleen, kidney, lung, erythrocytes, heart and skeletal muscle in that order. The results of their studies were obtained from the mature female rats under the normal physiological condition. It is also of interest to note that no significant differences were detected in $^{3}H/^{14}C$ ratios of all four tissues between the control and niacin deficient groups. However, the $^{3}H/^{14}C$ ratios of both control and niacin deficient groups were significantly ($p < 0.05$ or $p < 0.01$) higher than those of the food-restricted group. These observations suggest that there is a significant difference in protein turnover rates of the food-restricted group as compared to the other two groups but there is no difference between the other two groups. These gross observations, however, should be interpreted on the basis of individual isotope patterns.

In regard to ^{3}H incorporation in each tissue the dpm for ^{3}H in the food-restricted group was slightly lower

Table 41. $^{3}H/^{14}C$ Ratios of Total Proteins from Quail Tissues with 6 day Intervals Between Injections (Experiment 10)[1,2]

Diet	Brain			Heart		
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
	dpm x 10^{-5}/total brain			dpm x 10^{-5}/total heart		
NFD + 0.007% nicotinic acid (ad libitum)	3.23±0.20[a]	0.80±0.03[A]	4.04±0.11[AB]	3.62±0.34[A]	0.81±0.05[A]	4.47±0.23[A]
N F D (ad libitum)	3.54±0.46[a]	0.78±0.08[A]	4.54±0.40[A]	2.26±0.35[B]	0.47±0.06[B]	4.81±0.60[A]
NFD + 0.007% nicotinic acid (restricted)	3.29±0.42[a]	1.14±0.06[B]	2.89±0.30[B]	1.79±0.30[B]	0.75±0.05[A]	2.39±0.33[B]

Continued

[1]Values are mean ± S.E of 8 observations. Each of eight quail from each treatment received an intraperitoneal injection of 9.5 μCi of U-^{14}C-leucine. Six days later each quail received 68 μCi of [4,5-^{3}H]-leucine. Four hours after the last injection, the animals were killed and the total proteins were isolated and counted as described in the Experimental Procedures.

[2]Means not sharing a common superscript letter within a column were significantly different ($P<0.05$ or $P<0.01$).

Abbreviation: NFD, niacin free diet.

Table 41. Continued.

Diet	Liver			Breast muscle		
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	$^{3}H/^{14}C$
	dpm x 10^{-5}/total liver			dpm x 10^{-5}/total breast muscle		
NFD + 0.007% nicotinic acid (ad libitum)	41.85 ± 3.00^{A}	4.70 ± 0.61^{a}	8.90 ± 0.90^{a}	36.8 ± 2.74^{A}	16.8 ± 1.03^{A}	2.19 ± 0.12^{a}
N F D (ad libitum)	21.19 ± 2.98^{B}	2.43 ± 0.28^{a}	8.72 ± 1.30^{a}	10.49 ± 2.07^{B}	3.50 ± 0.53^{B}	3.00 ± 0.60^{Aa}
NFD + 0.007% nicotinic acid (restricted)	15.07 ± 2.88^{B}	3.54 ± 0.29^{a}	4.25 ± 0.90^{b}	9.85 ± 1.68^{B}	8.53 ± 0.83^{C}	1.15 ± 0.18^{Bb}

than the niacin deficient group whereas ^{14}C dpm of tissues in the food-restricted group was considerably ($p < 0.01$) higher than that of the niacin deficient group except for liver tissue. The lower ^{3}H dpm in the food-restricted group indicates a slightly lower rate of protein synthesis as compared to that in the niacin deficient group. However, the higher ^{14}C dpm obtained with the food-restricted group could be explained on the basis that protein degradation rates are reduced in the food-restricted group (particularly in breast muscle tissue) as compared to the niacin deficient group or that the reutilization of amino acids in the food-restricted group is much greater than in the niacin deficient group.

With respect to the effect of amino acid recycling on the protein turnover, the biochemical mechanisms responsible for this phenomenon are still poorly understood. Solomon and Tarver (1952) reported that recycling is a likely source of error due to an increase in amino acid reutilization under conditions of malnutrition, and is apt to result in overestimation of turnover rates. Labeled amino acids released by protein breakdown are likely to be reincorporated into new protein molecules without leaving the tissue (Waterlow and Stephen, 1968). Nettleton and Hegsted (1974) presented the experimental evidence that amino acid recycling is accentuated during protein deficiency and this can be minimized by feeding cold amino acids. Lane (1967) reported that the greater retention of labels in protein deficiency

was due to decrease in the catabolic rates of tissue proteins, as well as to increased recycling of amino acids. Gan and Jeffay (1967) also reported that 50% of the liver and 30% of the muscle amino acid pools in rats were derived from protein degradation during the short-term starvation and thus were available for recycling. This is a very effective adaptive mechanism during short-term starvation in the rat.

With respect to the current study it may be concluded that the niacin deficient and food-restricted quail were maintained in a semi-starved condition. It may be hypothesized that the degree of recycled amino acids in the food-restricted and niacin deficient groups would be similar to each other as they both consumed similar amounts of the same diet (except for nicotinic acid) and they had similar overall growth patterns.

However, the degree of amino acid recycling in these quail as indicated in the above literature discussion would be much greater than that of the control quail. This higher degree of recycling of amino acids would tend to increase ^{14}C values in the food-restricted group and as a result would yield a low apparent $^{3}H/^{14}C$ ratio. Under these conditions it is not possible to compare turnover rates of the control ad libitum fed group with the other two groups as the ratio would be influenced in a variable manner by the two factors.

It should be possible, however, to compare the other two groups (groups II and III) if it is assumed that the degree of recycled amino acids in both groups are similar. Under this condition any differences that occur between them is attributable to a differential rate of tissue degradation and synthesis as indicated by both the ^{14}C and ^{3}H values. If the data is interpreted on the basis of the above assumption it may be concluded that in all tissues (particularly breast muscle) the degradative rates of proteins in the niacin deficient quail are greater than those of the food-restricted quail.

With respect to protein synthesis rates it may be concluded on the basis of the ^{3}H data that the rate of synthesis in the niacin deficient quail is slightly higher than that of the food-restricted quail. Also, that the control ad libitum fed quail had a much higher rate of protein synthesis in all tissues except the brain relative to quail in the other two groups. These data indirectly support the idea that protein synthesis is reduced in fasting (Millward et al., 1973). The higher ^{3}H and ^{14}C values in the control group relative to the niacin deficient and food-restricted groups may result from a combination of concomitantly increased synthesis and decreased degradation of proteins. Ogata et al. (1978) suggested that nutritionally dependent growth depends both on stimulation of synthesis and depression of degradation of proteins.

The next series of experiments were designed to establish the relationship between subunit molecular weights or isoelectric points and protein turnover rates for the three groups of quail. A typical elution pattern of radioactivity from Sephadex G-200 of quail breast muscle proteins treated with SDS is illustrated in Figure 22. Studies with simultaneously injected isotopes demonstrated that the two isotopes were incorporated into breast muscle proteins of the control ad libitum group at the same rate (Figure 22A). The ratios from the other two groups also followed the same pattern.

A comparison of the relative turnover rates of breast muscle proteins for the three groups of quail (Figure 22B) indicates that there is a differential pattern among the various fractions and that the readily solubilizable proteins in NCS tissue solubilizer appear to be eluated at molecular weight values smaller than approximately 35,000.

As observed in the previous section, the average $^{3}H/^{14}C$ ratios of the niacin deficient and the control groups are higher than those of the food-restricted group. The overall average $^{3}H/^{14}C$ ratios for breast muscle proteins in this experiment (with SDS) and in the previous experiment (with TCA) were 2.67 and 2.19, 3.12 and 3.0, and 1.12 and 1.15, for the control, niacin deficient and food-restricted groups, respectively. The slight discrepancy may be attributed to the fact that the data from the current experiment represents average values of the ratio and not weight

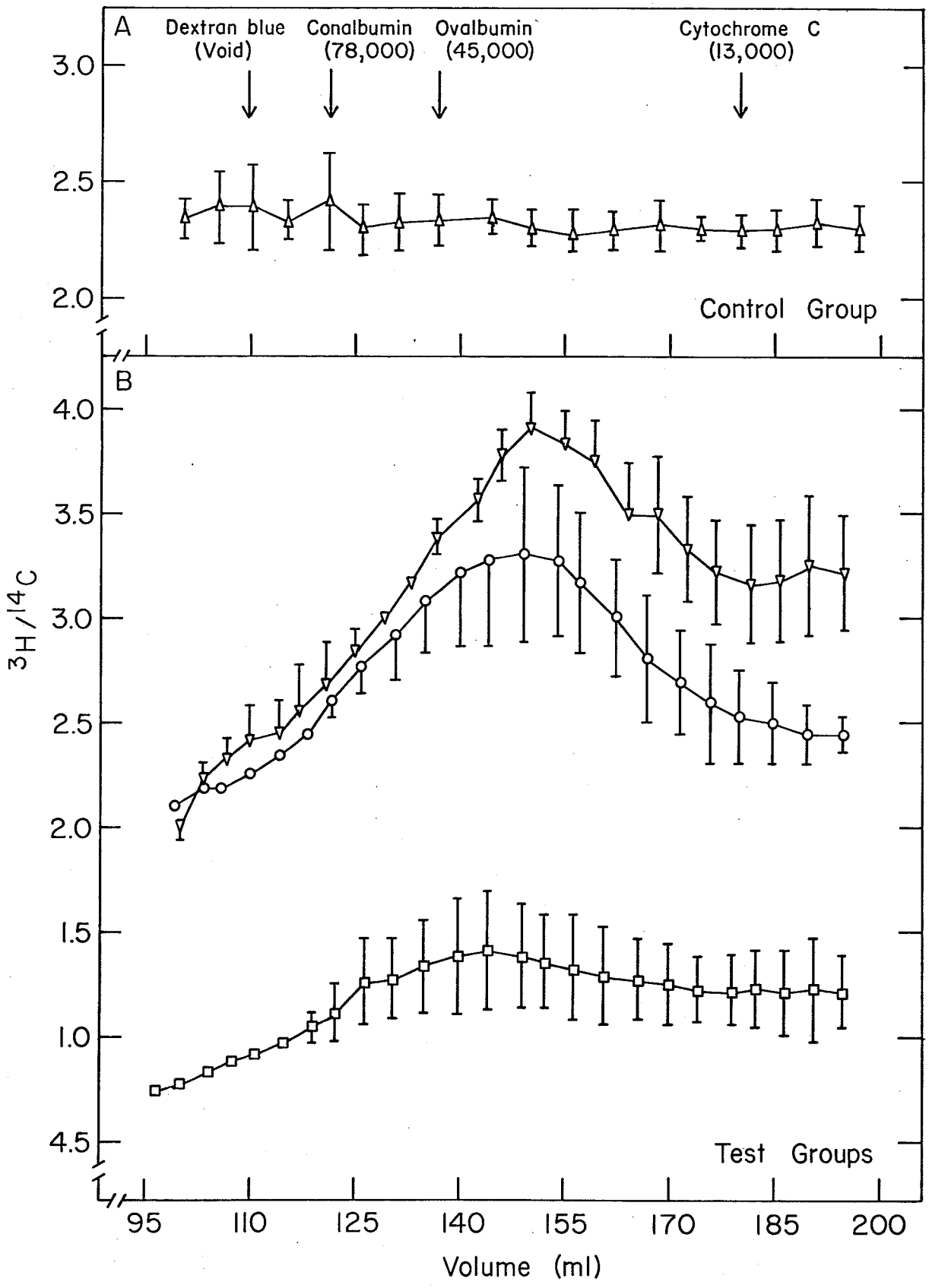
Figure 22. Fractionation of Quail Breast Muscle Proteins on Sephadex G-200 in the Presence of SDS (Experiment 10).

Double-labelled proteins were prepared from quail breast muscle as described in Experimental procedures. Four milliliter samples containing approximately 100-200 mg of protein were applied to a Sephadex G-200 column (2.5 x 70 cm). The radioactivity counting of protein samples was outlined in Experimental procedures. In the control group (A) breast muscle proteins from simultaneously injected quail were used. In the test groups (B) breast muscle proteins of quail (with 6 day intervals between injections) from three dietary treatments were used.

○ : NFD + 0.007% nicotinic acid (ad libitum)

▽ : NFD (ad libitum)

□ : NFD + 0.007% nicotinic acid (restricted)


A
Dextran blue (Void)
Conalbumin (78,000)
Ovalbumin (45,000)
Cytochrome C (13,000)
3.0
2.5
2.0
Control Group
B
4.0
3.5
3.0
2.5
2.0
1.5
1.0
4.5
Test Groups
$^3H/^{14}C$
95
110
125
140
155
170
185
200
Volume (ml)

average values. The results, nevertheless, are in remarkably close agreement. Part of these differences, particularly between the control and food-restricted groups, may be attributed, as discussed previously, to recycling of amino acids. The results from this study also indicate that there is both a negative and positive correlation between the size of subunits and the relative rates of protein turnover in all three treatment groups. In general, the $^{3}H/^{14}C$ ratios tend to increase as the subunit size decreases up to a molecular weight of slightly smaller than that of ovalbumin (M.W. 45,000) after which the ratios tend to decrease. The results of this experiment are somewhat different from those reported by Dehlinger and Schimke (1970). They found that there was a positive correlation between the size of a protein molecule, or more specifically the subunits of soluble proteins, of rat liver and their relative rates of degradation. This difference may be attributed to a difference in species, tissues, or age of animal. It should be emphasized, however, that molecular size is not the sole determinant of degradation, since three soluble proteins of rat liver, arginase (M.W. 118,000, 4 subunits) (Hirsch and Greenberg, 1969), tyrosine aminotransferase (M.W. 120,000, 3 subunits) (Valeriote et al., 1969), and LDH isozyme 5 (M.W. 136,000, 4 subunits) (Castellino and Barker, 1968), have half-lives of 4 days (Schimke, 1964), 1.5 hours (Kenney, 1967), and 16 days, respectively (Fritz et al., 1969). In addition, Dice et al. (1978) more

recently have reported that the relationship between protein size and degradative rate was absent among liver and muscle proteins from diabetic rats and that there was a reduced correlation between protein size and degradative rate in liver of the starved rats. These results would also suggest that dietary treatments had a general overall effect on most proteins rather than a specific effect on proteins of a certain molecular size.

A third experiment compared the relative turnover rates of proteins from the three groups that were separated on the basis of their isoelectric points. Typical electrophoretograms are given in Figure 23. The bulk of breast muscle proteins seemed to have pI between pH 5 and 8. All samples appeared to contain similar amounts of most proteins as evidenced by an examination of individual gels and the corresponding OD scan of the gel (Figure 24). The amount of background interference was the greatest in the food-restricted group (Figure 24C), followed by the niacin deficient group (Figure 24B) and the control ad libitum group (Figure 24A). This probably was attributed to a higher rate of denaturation of the protein from the first two groups during the process of electrophoresis. These patterns could be readily reproduced and the denaturation that occurred during electrophoresis also took place when the extracts of the samples were allowed to stand at either 0° or 20°C for several hours. These results would suggest that overall the proteins from the control ad libitum fed quail were more stable than those from the

Figure 23. Electrophoretograms of Breast Muscle Proteins from Three Dietary Treatments after Isoelectric Focusing in Polyacrylamide Gel (Experiment 10).

Approximately 10 mg of proteins were loaded onto each gel. See Experimental Procedures for further detail. Gel A, B, and C were from control, niacin deficient and food - restricted groups, respectively.

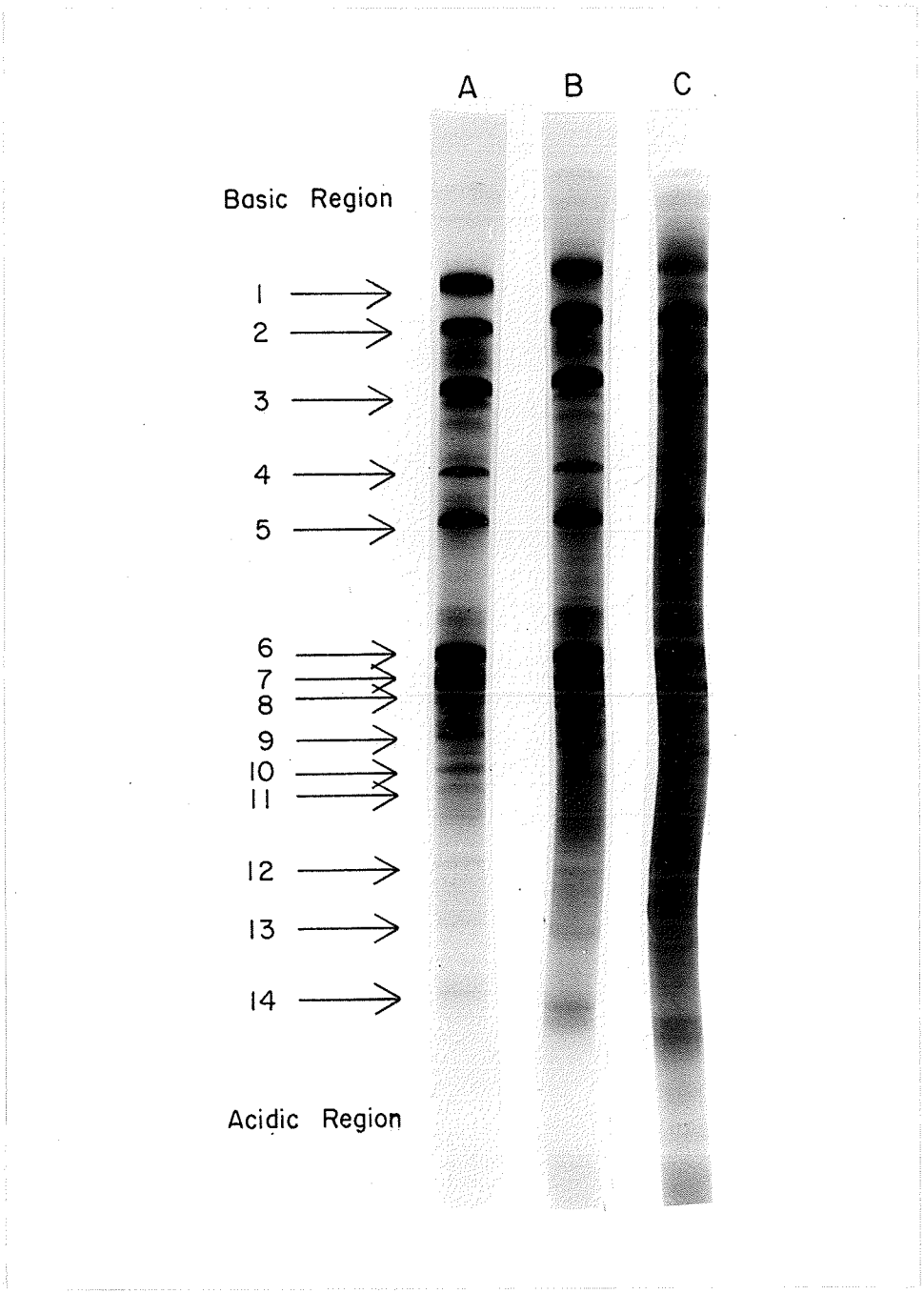

A
B
C
Basic Region
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Acidic Region

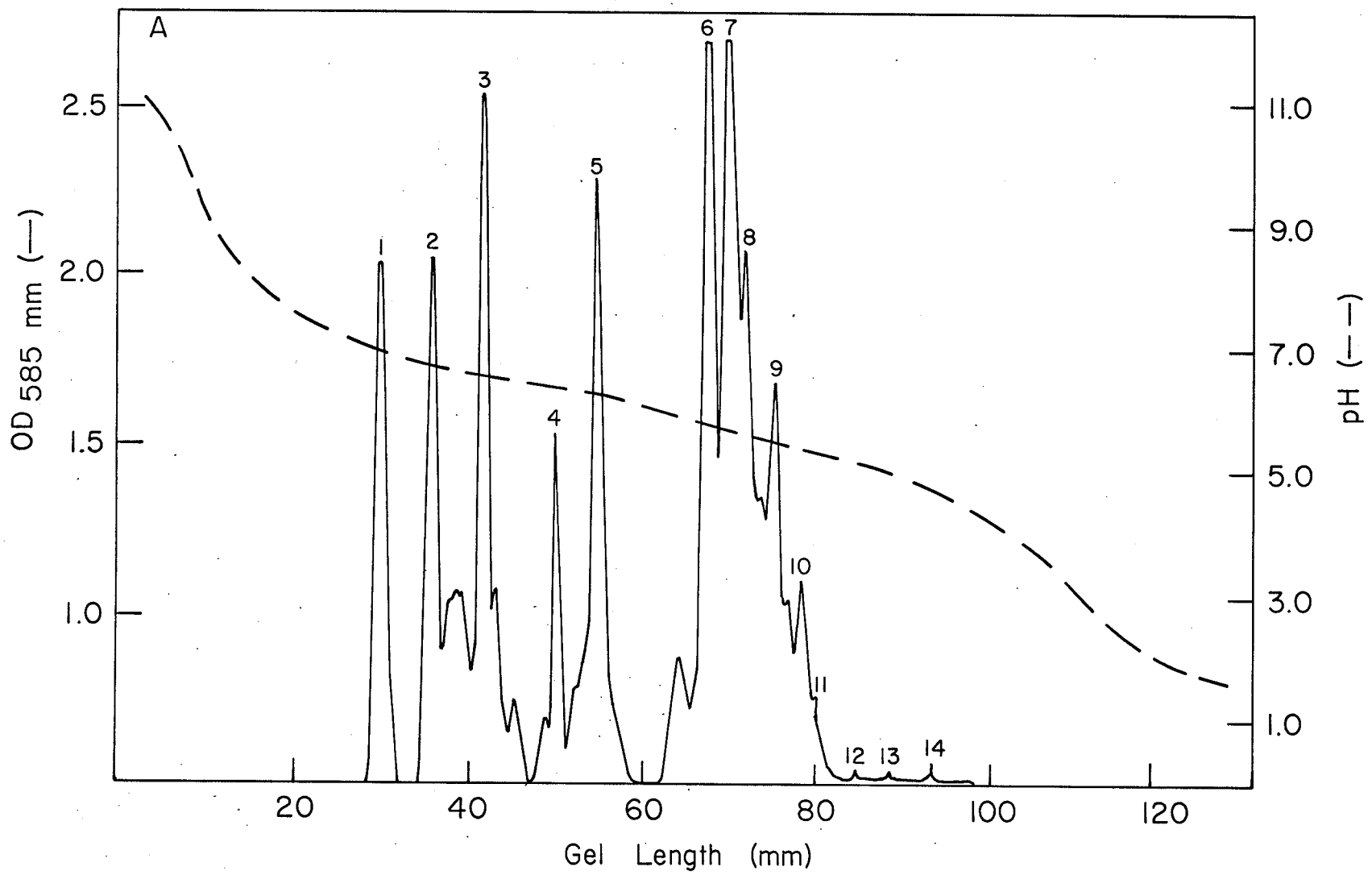
Figure 24. Optical Scan of Destained Gels of Breast Muscle Proteins Separated by Isoelectric Focusing in Polyacrylamide Gel (Experiment 10).

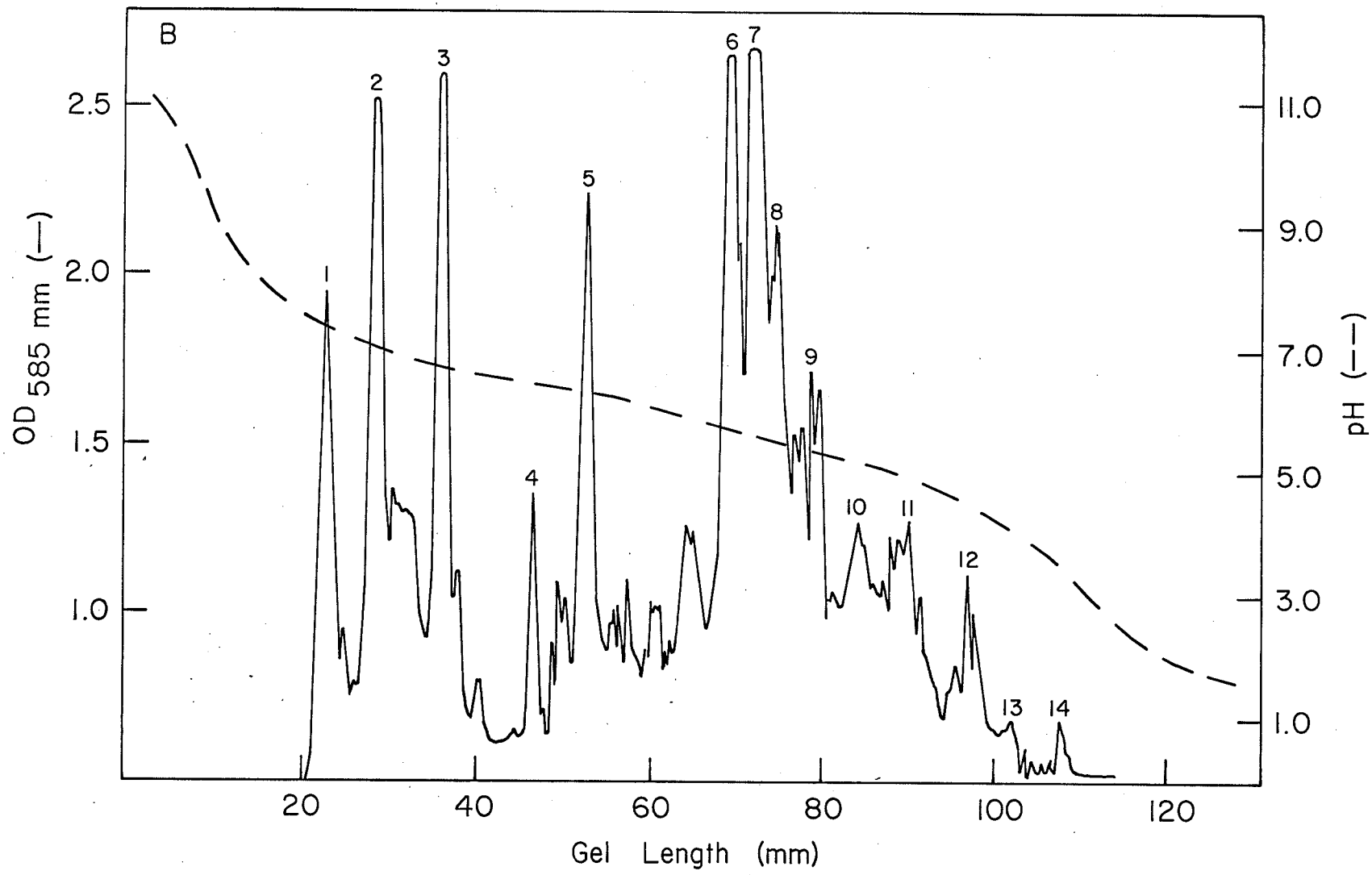
Approximately 10 mg of proteins were loaded onto each gel. See Experimental Procedures for further detail.

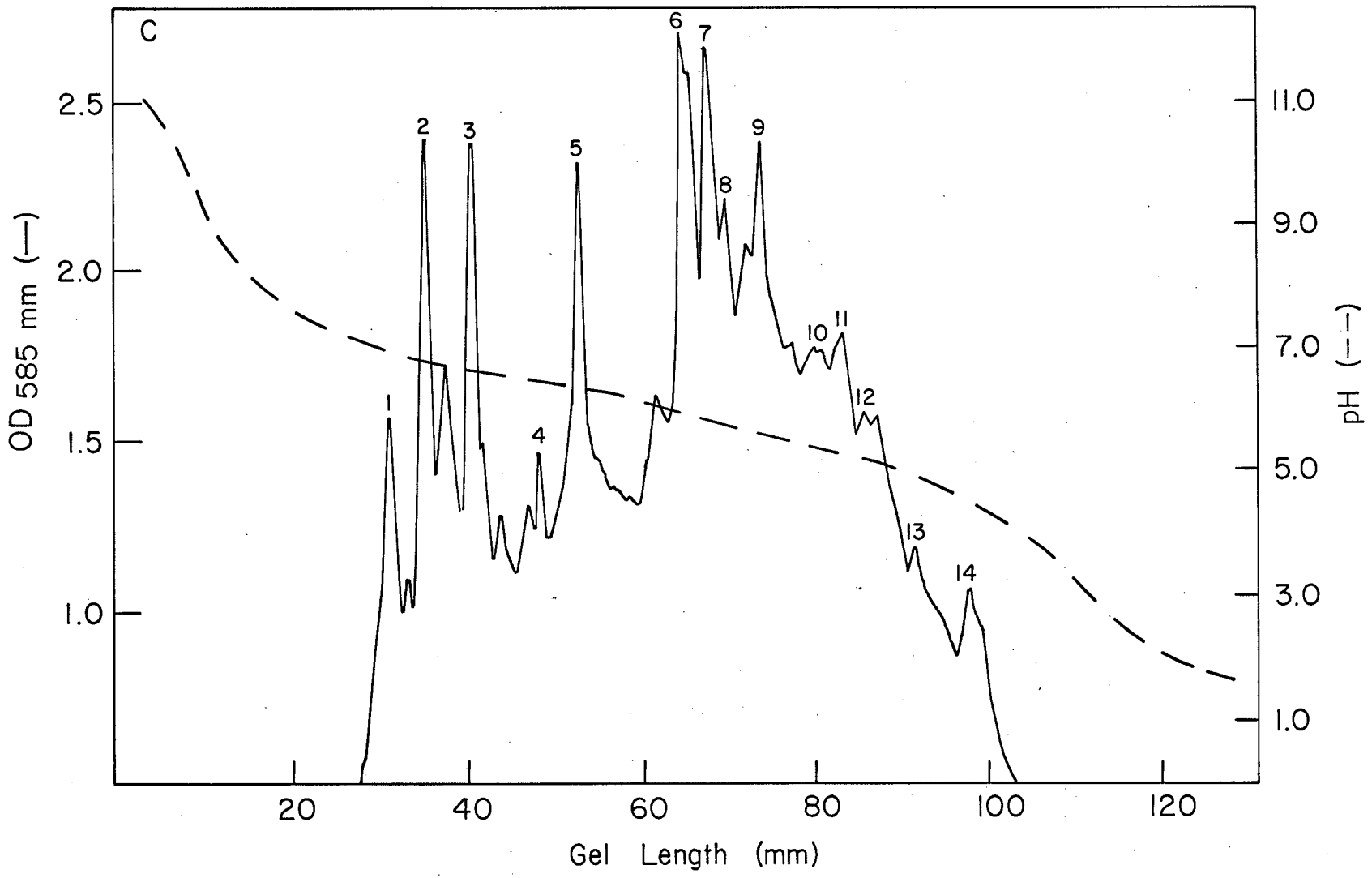
A : Control ad libitum group

B : Niacin deficient group

C : Food-restricted group


A
OD 585 mm (—)
pH (- - -)
Gel Length (mm)
2.5
2.0
1.5
1.0
11.0
9.0
7.0
5.0
3.0
1.0
20
40
60
80
100
120
1
2
3
4
5
6
7
8
9
10
11
12
13
14


B
OD 585 mm (—)
pH (—)
Gel Length (mm)
2.5
2.0
1.5
1.0
11.0
9.0
7.0
5.0
3.0
1.0
20
40
60
80
100
120
1
2
3
4
5
6
7
8
9
10
11
12
13
14


C
OD 585 mm (—)
pH (- -)
Gel Length (mm)
2.5
2.0
1.5
1.0
11.0
9.0
7.0
5.0
3.0
1.0
20
40
60
80
100
120
1
2
3
4
5
6
7
8
9
10
11
12
13
14

other two groups.

There also appeared to be a slightly different protein profile for the three groups with the niacin deficient group being different from the control and food-restricted groups. Several additional protein bands were observed between major protein bands 4 and 5, 5 and 6, and 8 and 9 in the niacin deficient group but were not present in the other two groups. These results would suggest that either there was a differential synthesis of certain proteins in the niacin deficient group or that protein bands 4, 5, and 8 of the other two groups may have dissociated into multiple bands in the niacin group due to denaturation. In addition, in the acidic fraction of the gel the protein bands 10, 12, 13 and 14 of the niacin deficient group consisted of a number of minor peaks. This may indicate a more pronounced lability of acidic proteins in the niacin deficient group. There did not appear to be any difference in the amount and location of individual proteins between the other two groups.

The changes in the $^{3}H/^{14}C$ ratios for breast muscle proteins separated by isoelectric focusing in polyacrylamide gels are illustrated in Figure 25. The correlation coefficient for the relationships between pI and $^{3}H/^{14}C$ ratios in the control simultaneously injected quail (Figure 25A) was $r = -0.29$ ($p > 0.05$). These results would suggest that under identical conditions the two isotopes would be incorporated into proteins in a similar manner.

Figure 25. Relative Degradative Rates of Breast Muscle Proteins Separated by Isoelectric Focusing in Polyacrylamide Gel (Experiment 10).

Approximately 10 mg of proteins were loaded onto each gel. See Experimental Procedures for further detail. In the control group(A) breast muscle proteins from simultaneously injected quail were used. In the test groups (B) breast muscle proteins of quail (with 6 day intervals between injections) from three dietary treatments were used.

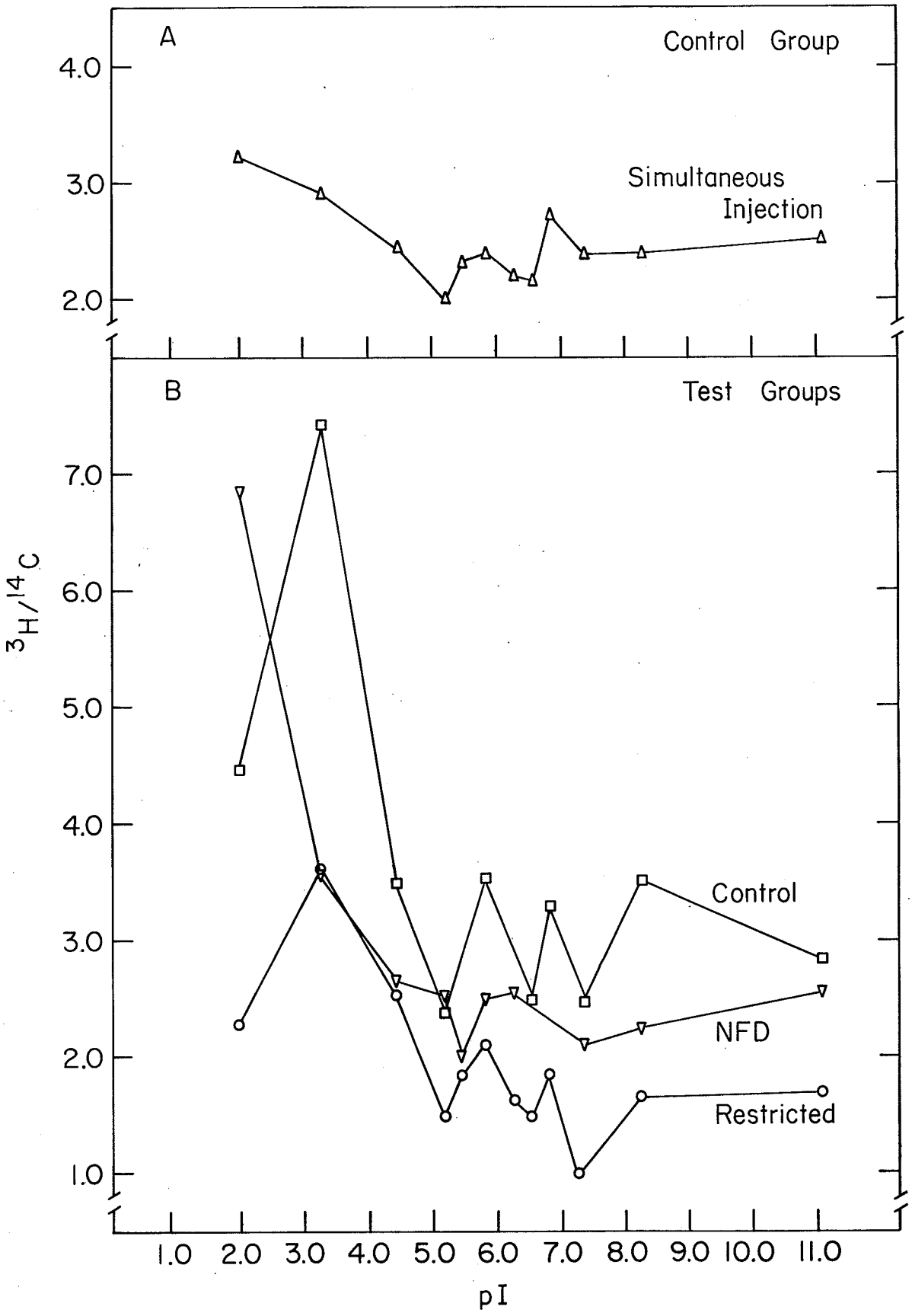

A
Control Group
Simultaneous Injection
4.0
3.0
2.0
B
Test Groups
Control
NFD
Restricted
7.0
6.0
5.0
4.0
3.0
2.0
1.0
$^{3}H/^{14}C$
1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0
pI

Figure 25B shows the comparative labeling patterns of the soluble proteins from breast muscle of the three groups following isoelectric focusing in polyacrylamide gels. The results would indicate that in all three treatment samples the acidic proteins tend to have higher $^{3}H/^{14}C$ ratios and, therefore, a higher rate of protein turnover than the neutral or basic proteins. The correlation coefficients and levels of statistical significance for the relationship between pI and $^{3}H/^{14}C$ ratios are as follows: control group, $r = -0.633$, $p < 0.01$; niacin deficient group, $r = -0.64$, $p < 0.01$; food-restricted group, $r = -0.77$, $p < 0.01$. Although the ratios of $^{3}H/^{14}C$ differ between the control and food-restricted quail the overall patterns are similar and almost superimposable. Part of the difference in $^{3}H/^{14}C$ ratios between these two groups may be attributed to the recycling effect of amino acids. These effects were discussed previously but would suggest that the most valid comparison would be between the niacin deficient and the food-restricted groups. The niacin deficient group in contrast to the food-restricted group has a different pattern particularly with regard to the $^{3}H/^{14}C$ ratio of proteins having a pI value of 2 and also those proteins having pI values between 5 and 7. The results would suggest that the degradative rates of these proteins may be greater in the niacin deficient quail as compared to quail in the other two groups.

Dice and Goldberg (1975b) reported that in normal rat liver, skeletal muscle, kidney, and brain, more rapid rates

of catabolism were found for acidic proteins than for neutral or basic ones and that there was a significant correlation between relative turnover rates and pI ($p < 0.01$). In relation to these findings, Duncan and Bond (1977) reported that acidic proteins from liver cytosol have larger subunits than do basic ones and would then have higher turnover rates. The biochemical mechanisms of the rapid degradation of acidic proteins is not fully understood yet. It is not even clear whether the protein net charge itself is the crucial physical parameter in influencing degradative rates, or whether a related property such as charge density may be the important factor (Momamy et al., 1976).

However, several possible explanations were put forward.

1) Acidic proteins may be especially susceptible to proteolytic attack only under specific conditions.
2) The cellular proteases actually responsible for intracellular protein degradation may preferentially hydrolyze the acidic proteins.
3) Acidic proteins might preferentially accumulate in regions of the cell where degradative enzymes are localized.

Thus acidic proteins may be taken up by lysosomes more rapidly or may bind to lysosomal membranes more readily (Dean, 1975) than neutral or basic proteins. Recent observations by Dice et al. (1978) indicate that the tendency of acidic proteins to turn over more rapidly is decreased or

abolished in liver and muscle of starved or diabetic animals. Thus the features of the degradative process that are responsible for the correlation between net charge and half-life must be masked or altered during the enhanced protein degradation seen in diabetes and starvation.

In the current study the general overall pattern for the control quail is in agreement with the observations of Dice et al. (1978) and Dice and Goldberg (1975b). Comparative analyses for the niacin deficient quail are not available in the literature but results from the current study would suggest that the general pattern may be modified in the niacin deficient quail.

In conclusion, the results of the current study would suggest that turnover rates of proteins in various tissues among the various dietary treatments are probably different and may be chiefly attributable to an altered rate of degradation rather than synthesis. In general, protein turnover rates in breast muscle tissue of the niacin deficient quail are particularly affected while the effect on other tissues is probably minimal. As shown in other studies the accurate measurement of protein turnover rates in non-steady conditions may be hampered by amino acid recycling. The effect of niacin deficiency on protein turnover appears to not only enhance degradative rates of proteins in general but also even greater degradative rates of certain specific enzymes or proteins which have an acidic pI. Isoelectric focusing for pI determination seems to provide a more effective and

meaningful method of resolving proteins than the molecular sieving technique.

CONCLUSIONS

Studies on the in vitro stability of pyridine nucleotides would demonstrate that under the conditions tested there is a very slow but progressive decline in pyridine nucleotide concentration. Factors that affect this would include the time after freezing, duration of the homogenization period and the amount of reducing reagents in the extracting media.

The results also demonstrate that it is difficult, if not impossible, to induce niacin deficiency in mature quail even when the synthesis of niacin by microorganisms is depressed by the administration of sulfaguanidine or when the metabolism of niacin is disturbed by the addition of 6-aminonicotinamide or a high amount of leucine to the diet. The results obtained with immature quail indicated that it is possible to induce niacin deficiency symptoms in these animals. The severity of the symptoms also appears to be age related being most severe in the very young bird.

Under conditions of severe niacin deficiency, there was a marked reduction in the level of NAD and NADPH in the breast muscle of immature quail. Pyridine nucleotide levels in other tissues such as liver, brain, and heart, however, were not influenced by niacin deficiency. These findings would suggest that NAD(H) or NADP(H) may be transported from the breast muscle tissue to other more essential organs under conditions of niacin deficiency. There was, however,

no apparently close association between the niacin status of quail and the corresponding activity levels of metabolically related dehydrogenases. In general, the breast muscle dehydrogenase activity levels of the niacin deficient birds were similar to those of the food-restricted birds whereas this relationship was not always observed with the liver enzymes.

Studies on the effects of dietary nicotinic acid or tryptophan levels and feeding pattern on the fate of 7-^{14}C-nicotinic acid in different tissues demonstrate that there was a significant threeway interaction among diets, tissues, and time in the metabolism of radioactive products following the injection of isotope. It was shown that the turnover rates of radioactive niacin metabolites in all tissues (particularly breast muscle) of the niacin deficient quail were extremely low as compared to other dietary treatments and that the rate of turnover varied among tissues. Chromatographic separation of the radioactive products demonstrated that most of the radioactivity was associated with nicotinamide, NAD, and NADP in liver and NAD and nicotinamide in breast muscle regardless of the level of niacin in the diet.

Thermal stability studies of enzymes <u>in vitro</u> showed that glyceraldehyde-3-phosphate dehydrogenase from breast muscle tissue of niacin deficient birds had the greatest sensitivity to heat inactivation relative to other dehydrogenases or non-dehydrogenases. It may be hypothesized that the low level of NAD in the breast muscle tissue of the

niacin deficient bird may have been directly responsible for the decreased enzyme stability or its deficiency may have indirectly caused the accumulation of certain tissue metabolites or proteases which in turn had a destabilizing effect on the enzyme. Taylor et al. (1948) reported that muscle glyceraldehyde-3-phosphate dehydrogenase cannot be crystallized without NAD and becomes very labile when the bound NAD is released. Deal (1969) indicated that NAD is essential in the "folding control" of translation of glyceraldehyde-3-phosphate dehydrogenase. It appears that NAD is necessary not only for the appearance of enzyme activation but also for stabilization of the conformation of glyceraldehyde-3-phosphate dehydrogenase molecule.

Studies of niacin deficiency on general protein turnover suggest that turnover rates of proteins in various tissues may be attributable to an altered rate of degradation rather than synthesis. In general, protein turnover rates in breast muscle and presumably other skeletal muscle tissues of the niacin deficient bird are markedly enhanced while the effect on other tissues is probably minimal. Isoelectric focusing in polyacrylamide gel showed that the breast muscle proteins from the niacin deficient group, in general, appeared to be more labile than those from the control and food-restricted groups. In all treatment samples the acidic proteins tended to have higher $^{3}H/^{14}C$ ratios and, therefore, a higher rate of protein turnover than the neutral or basic proteins. The niacin deficient quail in contrast

to the food-restricted group had a different pattern particularly with regard to the $^{3}H/^{14}C$ ratio of proteins having a pI value of 2 and also those proteins having pI values between 5 and 7. The results would suggest that the degradative rates of these acidic proteins may be greater in the niacin deficient quail as compared to quail in the other two groups.

The intricate nature of the effect of niacin deficiency would suggest that the primary consequences of these effects on overall metabolism should be further examined in various aspects. By what mechanism does the niacin deficient animal undergo muscle atrophy and how does it induce symptoms such as blindness? The search for answers to such questions has to be explored in future research. The most important question regarding the effect of niacin deficiency in animal lies in establishing the specific manner that niacin deficiency affects the overall metabolism. Therefore, the following areas of research are suggested for future investigation. To this end, most of this study should be carried out with muscle tissue as it appears to be the tissue that is mainly affected by niacin deficiency.

(1) Determination of the Level of RNA, DNA, and Poly (ADP-ribose) and NADase Activity

It has been suggested that the physiological role of NADase is to regulate the rate of certain metabolic processes through control of the levels of required oxidized nico-

tinamide coenzymes (Kaplan, 1966). The observation that diphtheria toxin catalyzes the NAD-dependent ADP ribosylation of elongation factor II (Honjo et al., 1971), thus inhibiting protein synthesis, and that A protomer of choleragen (Moss et al., 1976) has marked NADase activity provides support for additional regulatory roles for these enzymes. The effect of poly (ADP-ribose) formed from NAD by poly (ADP-ribose) synthetase on DNA synthesis is not well established yet. However, Roberts et al. (1973) reported that the effect of poly (ADP) ribosylation on DNA synthesis is apparently dependent on the type of cells involved. Indirect evidence nevertheless suggested that poly (ADP-ribose) in some manner regulates DNA synthesis, DNA repair, RNA metabolism, cell division or differentiation (Hilz and Stone, 1976). Poly (ADP-ribose) synthetase may be important in vivo in the control of the tissue concentration of the nicotinamide nucleotide (Nishizuka et al., 1971). It was once thought that poly (ADP-ribose) might be a reservoir of NAD, but the reverse reaction, which should yield NAD from poly (ADP-ribose) and nicotinamide, has not been observed (Nishizuka et al., 1967). It is still possible that nicotinic acid can push the reverse reaction to produce deamido-NAD from poly (ADP-ribose). Deamido-NAD could not be a substrate for polymerization so that it could be reutilized to form NAD if the reverse reaction with nicotinic acid was possible (Haines et al., 1969). It has been proposed that poly (ADP-ribose) is in dynamic equilibrium with NAD pathways (Rechsteiner et al., 1976), but a precursor-product type

study, connecting NAD turnover to poly (ADP-ribose) has not been carried out in intact animals. Therefore, it is of interest to establish the relationship between the level of poly (ADP-ribose) and NAD turnover as well as the effect of poly (ADP-ribose) on RNA and DNA metabolism in relation to protein turnover rates in the niacin deficient animals.

(2) Determination of Leucine

The metabolism of leucine in muscle is of special interest since this branched chain amino acid, and no other one, may play a pivotal role in regulation of protein turnover in skeletal muscle (Buse and Reid, 1975). They reported that leucine inhibits protein degradation and promotes protein synthesis in rat skeletal muscle. Therefore, they proposed that the protein turnover in muscle may be regulated by the concentration of leucine in this tissue which may be varied under various nutritional and hormonal conditions. In accord with this view, Atwell et al. (1977), have shown that leucine increases polysome formation and reduces the number of free ribosomes in muscle. It is presently unknown whether the critical factor regulating protein turnover is leucine itself, some product of leucine degradation, or some other metabolite such as leucyl-tRNA. Since the level of charged t-RNA is known to regulate protein breakdown in bacteria, Goldberg and Chang (1978) postulated that the concentration of leucyl-tRNA may affect protein degradation in muscle tissue which rapidly cata-

bolizes this branched chain amino acid.

(3) Determination of Polysomal Profiles

In livers of fasted rats, Henshaw et al. (1971) found not only a decreased ribosome content but also a lower polysome activity and a lower proportion of ribosomes in polysomes. Also Stirewalt et al. (1968) showed that there was a marked disaggregation of polysomes in skeletal muscle of the fasting rat. It should be examined whether the changes in polysomal profiles in skeletal muscle in response to severe niacin deficiency is similar to that which occurs during fasting or may be due to alterations in the synthesis of mRNA, which might influence the state of the polysomal aggregates.

(4) Determination of Tryptophan

Tryptophan is a precursor of NAD (Chaykin, 1967), inhibits gluconeogenesis (Veneziale et al., 1967), increases fatty acid synthesis (Sakurai et al., 1973), and polysomal aggregation (Wunner et al., 1966). Murty et al. (1976) suggested that tryptophan may act to stimulate the transfer of poly A-containing mRNA from nucleus to cytoplasm and thus may probably play an important role at the post-transcriptional step in regulating the level of cytoplasmic mRNA in the liver.

(5) In Vitro Polypeptide Synthesis

The method of Loeb (1970) which was originally developed by Nirenberg (1961) can be used to study the effect of excess poly U on the rate of ^{14}C-phenylalanine incorporation into TCA-insoluble precipitates by liver microsomes from niacin fed and niacin deficient quail.

(6) Determination of the Level of NAD-specific Group Proteases

The activity of these enzymes has been known to be increased in niacin deficiency which may inactivate the apoform of dehydrogenases (Katunuma et al., 1971). Further research should be carried out to establish if specific proteases are involved in the degradation of dehydrogenases and the role of pyridine nucleotides as inhibitors of these proteins.

(7) The Synthesis and Degradation of Glyceraldehyde-3-Phosphate Dehydrogenase In Vivo

The relative turnover rates for general and specific proteins can be measured by a double-isotope technique (Arias et al., 1969) and the degradation rate in vivo of enzyme can be determined by means of single pulse labeling with $NaH^{14}CO_3$ (Millward, 1970) or other label and by following the disappearance of ^{14}C from the enzyme protein. The labeled glyceraldehyde-3-phosphate dehydrogenase can be isolated using general ligand affinity chromatography (Bachman

and Lee, 1976) or immunoprecipitation in a manner similar to that employed by Schimke et al. (1965) with other enzymes.

(8) Establish Comparative Stabilities of Dehydrogenases and Related Enzymes in the Presence of Varying Concentrations of Pyridine Nucleotides

Studies such as these should provide additional valuable information on the influence of niacin deficiency on enzyme stability which may, in turn, be related to protein turnover rates.

(9) Establish Rate Limiting Reaction in the Muscle Tissue

Assay of metabolite levels in the control and niacin deficient quail would assist in identifying crossover points or points at which metabolic blockages occur.

(10) Characterization of Certain Protein Bands Isolated from pI Study, Particularly Those That Appear to be Affected by Niacin Deficiency

(11) Development of Methods for Reducing or Minimizing Amino Acid Recycling Without Increasing the Levels of Amino Acids or Proteins in the Diet

Amino acid recycling is particularly serious when normal physiological conditions are perturbed by a drastic stress such as starvation or vitamin deficiency. A variety of experimental approaches have been used to minimize the re-

cycling of radioactive residues. In this context, the proper choice of radioactive precursors which reduce the extent of recycling is highly desirable since the physiological function or metabolic rate of different amino acids may be different in different tissues. The use of ^{14}C-carbonate as a precursor was considered to minimize the recycling of label (Millward, 1970). In addition to arginine, the ^{14}C from carbonate also appear in the carboxyl groups of glutamate and aspartate. The radioactivity of these two amino acids declines rapidly because of decarboxylation that occurs during glutamate and aspartate interconversion in the TCA cycle. The $H^{14}CO_3^-$ produced in this way is diluted by a large and freely diffusible pool of intracellular bicarbonate; consequently, the chances of reutilization of $^{14}CO_2$ released by amino acid degradation should be greatly reduced. Procedures in which the probability of recycling of the radioactive product of protein degradation is lowest are those in which the tracer is incorporated into the protein molecule after its translation. If the product of post-translational modification is a single amino acid (i.e., if the tracer is not metabolized), there is no tracer reutilization. The best examples of such cases are hydroxylation of proline in protocollagen (Lindstedt and Prockop, 1961) and methylation of histidine in myosin and actin (Funabiki et al., 1976). Since 3-methylhistidine is quantitatively excreted (Young et al., 1972), one can follow the turnover of muscle proteins by measuring the radioactivity excreted

(Nishizawa et al., 1977). An advantage of this method is that it can be applied to the study of muscle turnover in humans as well as in experimental animals. A disadvantage of this method is that the relative concentration of this amino acid in muscle proteins is very low (Huszar and Elzinger, 1971).

A study of the above suggested proposals could perhaps explain the mode by which niacin deficiency affects animal performance.

BIBLIOGRAPHY

Abeles, R.H., Hutton, R.F., and Westheimer, F.H. (1957) J. Am. Chem. Soc. 79:712.

Abeles, R.H., and Westheimer, F.H. (1958) J. Am. Chem. Soc. 80:5459.

Adams, R.L., and Carrick, C.W. (1967) Poult. Sci. 46:712.

Adler, E., Hellström, H., and von Euler, H. (1936) Z. Physiol. Chem. Hoppe-Seyler's. 242:225.

Albus, H., Schlenk, F., and von Euler, H. (1936) Biochem. Z. 286:140.

Allen, N.K., Baker, D.H., and Graber, G. (1971) Poult. Sci. 50:1540.

Almquist, H.J., and Mecchi, E. (1941) Proc. Soc. Exp. Biol. Med. 48:526.

Alpers, D.H. (1972) J. Clin. Invest. 51:2621.

Anderson, E.G., Teply, L.J., and Elvehjem, C.A. (1944) Arch. Biochem. 3:357.

Arias, I.M., Doyle, D., and Schimke, R.T. (1969) J. Biol. Chem. 244:3303.

Atkinson, D.E. (1977) Cellular Energy Metabolism and Its Regulation, Acad. Press, N.Y.

Atwell, J.R., Hedden, M.P., Mancusi, V.J., and Buse, M.G. (1977) Diabetes. 26 (Suppl. 1) :Abst. 81.

Axelord, A.E., Madden, R.J., and Elvehjem, C.A. (1939) J. Biol. Chem. 131:85.

Axelord, A.E., Spies, T.D., and Elvehjem, C.A. (1941) J. Biol. Chem. 138:667.

Bachman, B.K., and Lee, C.Y. (1976) Anal. Biochem. 72:153.

Ballard, F.J. (1977) Essays Biochem. 13:1.

Ballard, F.J., Hopgood, M.F., Reshef, L., and Hanson, R.W. (1974) Biochem. J. 140:531.

Bapurao, S. (1975) Ph.D. Thesis, Osmania Univ., India.

Barrett, A.J. (1972) In "Lysosomes" (Dingle, J.T., ed.) p. 46, American Elsevier Publishing Co., New York.

Beadle, G.W., Mitchell, M.K., and Nyc, J.F. (1947) Proc. Natl. Acad. Sci. U.S. 33:155.

Beher, W.T., Holliday, W.M., and Gaebler, O.H. (1952) J. Biol. Chem. 198:573.

Beisenherz, G., Bucher, T., and Garbage, K.H. (1955) In "Methods in Enzymology" (Colowick, S.P., and Kaplan, N.O., eds.) Vol. I, p. 391, Acad. Press, New York.

Belavady, B., and Gopalan, C. (1966) Ind. J. Biochem. 3:44.

Belavady, B., Srinkantia, S.G., and Gopalan, C. (1963) Biochem. J. 87:652.

Bergström, J., Fürst, P., Norée, L.O., and Vinnars, E. (1974) J. Appl. Physiol. 36:693.

Biley, J., and March, B. (1951) Science. 114:330.

Black, S., McKibbin, J.M., and Elvehjem, C.A. (1947) Proc. Soc. Exp. Biol. Med. 47:308.

Black, S., Overman, R.S., Elvehjem, C.A., and Link, K.P. (1942) J. Biol. Chem. 145:137.

Blackhard, W.G., Nelson, N.C., and Andrews, S.S. (1974) Diabetes. 23:199.

Bohley, P., Hanson, H., Kirschke, H., Langner, J., Wiederandes, B., and Ansorge, S. (1976) In "Intracellular Protein Catabolism" (Turk, V., ed.) 2nd Int. Symp., J. Stefan Inst., Ljubljana, Yugoslavia. In Press.

Bohley, P., Langner, J., Ansorge, S., Miehe, M., Miehe, C., and Hanson, H. (1972) Acta. Biol. Med. Ger. 28:321.

Bokman, A.H., and Schweigert, B.S. (1951) Arch. Biochem. Biophys. 33:270.

Bond, J.S. (1971) Biochem. Biophys. Res. Commun. 43:333.

Bond, J.S. (1975) Fed. Proc. 34:651.

Bonner, D.M. (1948) Proc. Natl. Acad. Sci. U.S. 34:5.

Bradley, M.O., Dice, J.F., Hayflick, L., and Schimke, R.T. (1976) Exp. Cell. Res. 96:103.

Briggs, G.M. (1946) J. Nutr. 31:79.

Briggs, G.M., Groschke, A.C., and Lillie, R.J. (1946) J. Nutr. 32:659.

Briggs, G.M., Luckey, T.D., Teply, L.J., and Elvehjem, C.A. (1943) J. Biol. Chem. 148:517.

Briggs, G.M., Mills, R.C., Elvehjem, C.A., and Hart, E.B. (1942) Proc. Soc. Exp. Biol. Med. 51:59.

Briggs, G.M., Singal, S.A., and Sydenstricker, V.P. (1945) J. Nutr. 29:331.

Brin, M., and Thiele, V.F. (1967) J. Nutr. 93:213.

Brown, F.C. (1964) Can. J. Biochem. 42:489.

Brown, J.L. (1979) J. Biol. Chem. 254:1447.

Brown, J.L., and Roberts, W.K. (1976) J. Biol. Chem. 251: 1009.

Bruchhausen, F.V. (1964) Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak. 247:87.

Bruchhausen, F.V., and Herken, H. (1966) Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak. 254:388.

Brunnemann, A., Coper, H., and Herken, H. (1963) Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak. 245:541.

Buniva (1805 - 1808) Memories de l'Academie Imperiale des Sciences, Literature et Beaux-arts de Turin.

Burch, H.B., Bradley, M.E., and Lowry, O.H. (1967) J. Biol. Chem. 242:4546.

Burch, H.B., Storvick, C.A., Bicknell, R.L., Lowry, O.H., King, C.G., and Bessey, O.A. (1955) J. Biol. Chem. 212:897.

Burton, R.M., Kaplan, N.O., Goldin, A., Leitenberg, M., Humphreys, S.R., and Sodd, M.A. (1958) Science. 127:30.

Burzio, L., and Koide, S.S. (1970) Biochem. Biophys. Res. Commun. 40:1013.

Buse, M.G., Atwell, R., and Mancusi, V. (1979) Horm. Metab. Res. 11:289.

Buse, M.G., and Reid, S.S. (1975) J. Clin. Invest. 56:1250.

Callahan, J.W., and Kosicki, G.W. (1967) Can. J. Biochem. 45:839.

Cantoni, G.L. (1953) J. Biol. Chem. 204:403.

Caplan, A.I. (1976) In. Proc. 4th Int. Symp. "Poly (ADP-ribose) and ADP-ribosylation of Proteins", ed. H. Hilz. p. 8.

Carlson, C.A., and Kim, K.H. (1974a) Arch. Biochem. Biophys. 164:478.

Carlson, C.A., and Kim, K.H. (1974b) Arch. Biochem. Biophys. 164:490.

Carlson, L.A., Orö, L., and Östman, J. (1968) Acta. Med. Scand. 183:457.

Casal, D.G. (1762) Historia natural y medica de el Principado de Asturias, Madrid, p. 327.

Castellino, F.J., and Barker, R. (1968) Biochem. 7:2207.

Cerri, G. (1919) In "Pellagra" (Harris, H.F., ed.) Macmillan, New York.

Chaloupka, M.M., Williams, J.N., Reynolds, M.S., and Elvehjem, C.A. (1957) J. Nutr. 63:361.

Chambon, P. (1971) Ann. Rev. Biochem. 44:613.

Chambon, P., Weill, J., and Mandel, P. (1963) Biochem. Biophys. Res. Commun. 11:39.

Chance, B., and Park, H.J. (1967) J. Biol. Chem. 242:5093.

Chang, T.W., and Goldberg, A.L. (1978a) J. Biol. Chem. 253: 3677.

Chang, T.W., and Goldberg, A.L. (1978b) J. Biol. Chem. 253: 3696.

Chaudhuri, C.R., and Chatterjee, I.B. (1969) Science. 164: 435.

Chaykin, S. (1967) Ann. Rev. Biochem. 36:149.

Chaykin, S., Dagani, M., Johnson, L., and Samli, M. (1965) J. Biol. Chem. 240:932.

Chen, R.F., and Plaut, G.W.E. (1962) Fed. Proc. 21:244.

Childs, G.R., Carrick, C.W., and Hauge, S.W. (1952) Poult. Sci. 31:551.

Childson, O.P., Kitto, G.B., Pudles, J., and Kaplan, N.O. (1966) J. Biol. Chem. 241:2431.

Cho-Chung, Y.S., and Pitot, H.C. (1967) J. Biol. Chem. 242: 1192.

Citri, N. (1973) Adv. Enzymol. 37:397.

Clark, J.L., and Fuller, J.L. (1976) Eur. J. Biochem. 67: 303.

Clark, M.G., Kneer, N.M., Bosch, A.L., and Lardy, H.A. (1974) J. Biol. Chem. 249:5695.

Coates, M.E., Dickinson, C.D., Harrison, G.F., and Kon, S.K. (1951) Biochem. J. 49:68.

Coffey, J.W., and de Duve, C. (1968) J. Biol. Chem. 243: 3255.

Collier, R., and Pappenheimer, A.M. (1964) J. Exp. Med. 120:1019.

Colman, R.F., and Black, S. (1965) J. Biol. Chem. 240:1796.

Colman, R.F., and Frieden, C. (1966) J. Biol. Chem. 241: 3661.

Constantinides, S.M., and Deal, W.C. (1969) J. Biol. Chem. 244:5695.

Conway, A., and Koshland, D.E. (1968) Biochem. 7:4011.

Coper, H., Hadass, H., and Lison, H. (1966) Naunyn-Schmiedebergs. Arch. Pharmak. 255:97.

Coper, H., and Neubert, D. (1964) Biochim. Biophys. Acta. 89:23.

Cuendet, G.S., Loten, E.G., Cameron, D.P., Renold, A.E., and Marliss, E.B. (1975) Am. J. Physiol. 228:276.

Dann, W.J. (1941) J. Biol. Chem. 141:803.

Dann, W.J., and Handler, P. (1941) J. Biol. Chem. 140:935.

Dann, W.J., and Handler, P. (1942) J. Nutr. 24:153.

Dann, W.J., and Huff, J.W. (1947) J. Biol. Chem. 168:121.

Dann, W.J., and Kohn, H.I. (1940) J. Biol. Chem. 136:435.

Darby, W.J., McNutt, K.W., and Todhunter, E.N. (1975) Nutr. Rev. 33:289.

Davis, G.K., Freeman, V.A., and Madsen, L.L. (1940) Mich. State Agric. Exp. Stat. Tech. Bull. p. 170.

Deal, W.C. (1969) Biochem. 8:2795.

Deal, W.C., and Constantinide, S.M. (1970) Fed. Proc. Fed. Amer. Soc. Expt. Biol. 26:348.

Dean, R.T. (1975) Biochem. Biophys. Res. Commun. 67:604.

de Duve, C., and Wattiaux,R. (1966) Ann. Rev. Physio. 28: 435.

Deguchi, T., Arata, J., Nishizuka, Y., and Hayaishi, O. (1968) Biochim. Biophys. Acta. 158:382.

Dehlinger, P.J., and Schimke, R.T. (1970) Biochem. Biophys. Res. Commun. 40:1473.

DeMoss, R.D. (1955) In "Methods in Enzymology" (Colowick, S.P., and Kaplan, N.O., eds.) Vol. I, p.328, Acad. Press. New York.

Deter, R.L. (1971) J. Cell. Biol. 48:473.

De Vijlder, J.J.M., Boers, W., Hilvers, A.G., Harmsen, B.J.M., and Slater, E.C. (1969) In "Pyridine Nucleotide dependent Dehydrogenase" (Sund, H., ed.) p. 233, Springer-Verlag.

Dice, J.F., and Goldberg, A.L. (1975a) Arch. Biochem. Biophys. 170:213.

Dice, J.F., and Goldberg, A.L. (1975b) Proc. Natl. Acad. Sci. U.S.A. 72:3893.

Dice, J.F., Hess, E.J., and Goldberg, A.L. (1979) Biochem. J. 178:305

Dice, J.F., and Schimke. R.T. (1972) J. Biol. Chem. 247:98.

Dice, J.F., and Schimke, R.T. (1973) Arch. Biochem. Biophys. 158:97.

Dice, J.F., Walker, C.D., Byrne, B., and Cardiel, A. (1978) Proc. Natl. Acad. Sci. U.S.A. 75:2093.

Dietrich, L.S. (1971) Am. J. Clin. Nutr. 24:800.

Dietrich, L.S., Frieland, I.M.,and Kaplan, L.A. (1958a) J. Biol. Chem. 233:964.

Dietrich, L.S., Fuller, L., Yero, J.L., and Martinez, L. (1966) J. Biol. Chem. 241:188.

Dietrich, L.S., Kaplan, L.A., Frieland, I.M., and Martin, D.S. (1958b) Cancer. Res. 18:1272.

Dietrich, L.S., Muniz, O., and Powanda, M. (1968) J. Vitaminol. 14:123.

Dixon, W.J., and Brown, M.B. (1977) In "Biomedical Computer Programs P-Series" p. 230, Univ. California Press, Berkeley, L.A.

Don, M., and Masters, C.J. (1976) Int. J. Biochem. 7:215.

Duncan, W.E., and Bond, J.S. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36:918.

Duncan, M., and Sarett, H.P. (1951) J. Biol. Chem. 193:317.

Dus, K. (1976) In "Enzymes of Biological Membranes" (Martonosi, A., ed.) Vol. 4, p. 199.

Ellinger, P., Benesch, R., and Kay, W.W. (1945) Lancet. 248:432.

Ellinger, P., and Kader, M.M.A. (1949) Biochem. J. 45:276.

Elvehjem, C.A., Madden, R.J., Strong, F.M., and Woolley, D.W. (1937) J. Amer. Chem. Soc. 59:1767.

Engelhard, M., Rudolph, R., and Jaenicke, R. (1976) Eur. J. Biochem. 67:447.

Etlinger, J.L., and Goldberg, A.L. (1976) Fed. Proc. In Press.

Exton, J.H., and Park, C.R. (1966) Pharmacol. Rev. 18:181.

Feigelson, P., and Greengard, O. (1962) J. Biol.Chem. 237: 3714.

Feigelson, P., Williams, J.N., Jr., and Elvehjem, C.A. (1951) J. Biol. Chem. 193:737.

Fenselau, A. (1970) Biochim. Biophys. Res. Commun. 40:481.

Fisher, H., Scott, H.M., and Johnson, B.C. (1954) Poult. Sci. 33:1054.

Fitch, W.M., and Chaikoff, I.L. (1961) Biochem. 94:380.

Floyd, J.C., Jr., Fajans, S.S., Conn, J.W., Knoff, R.F., and Rull, J. (1966) J. Clin. Invest. 45:1487.

Frapolli, F. (1771) In "Morbium Vulgo Pelagram", Milan.

Friedland, I.M., Kaplan, L.A., Dietrich, L.S., and Shapiro, D.M. (1958) Fed. Proc. 17:224.

Friedman, B., Goodman, E.H., and Weinhouse, S. (1965) J. Biol. Chem. 240:3729.

Fritz, P.J. (1965) Science. 150:364.

Fritz, P.J., Vesell, E.S., White, E.L., and Puritt, K.M. (1969) Proc. Natl. Acad. Sci. U.S.A. 62:558.

Fritz, P.J., White, E.L., and Vesell, E.S. (1970) Fed. Proc. 29:735.

Fulks, R., Li, J.B., and Goldberg, A.L. (1975) J. Biol. Chem. 250:290.

Funabiki, R., Watanabe, Y., Nishizawa, N., and Hareyama, S. (1976) Biochim. Biophys. Acta. 451:143.

Funk, C. (1912) J. State. Med. 20:341.

Funk, C. (1922) In "The Vitamins" Williams and Wilkins, Baltimore.

Gaebler, O.H., and Beher, W.T. (1951) J. Biol. Chem. 188: 343.

Gan, J.C., and Jeffay, H. (1971) Biochim. Biophys. Acta. 252:125.

Gangadharam, P.R.J., Harold, F.M., and Schaefer, W.B. (1963) Nature. 198:712.

Garcia-Bunuel, L., McDougal, D.B., Jr., Burch, H.B., Jones, E.M., and Touhill, E. (1962) J. Neurochem. 9:589.

Garlick, P.J., Millward, D.J., James, W.P.T., and Waterlow, J.C. (1975) Biochim. Biophys. Acta. 414:71.

Gay, J.C., and Jeffay, H. (1967) Biochim. Biophys. Acta. 148:448.

Gay, J.C., and Jeffay, H. (1971) Biochim. Biophys. Acta. 252:125.

Gerber, G.B., and Deroo, J. (1969) Int. J. Radiat. Biol. 15:425.

Gerber, G.B., and Deroo, J. (1970) Proc. Soc. Exp. Biol. Med. 134:689.

Ghafoorunissa and Rao, N.B.S. (1973) Biochem. J. 134:425.

Gholson, R.K. (1966) Nature. 212:933.

Gholson, R.K., Ueda, I., Ogasawara, N., and Henderson,L.M. (1964) J. Biol. Chem. 239:1208.

Ginoulhiac, E., Tenconi, L.T., and Chiancone, F.M. (1962) Nature. 193:948.

Glock, G.E., and McLean, P. (1955) Biochem. J. 61:388 and 397.

Glock, G.E., and McLean, P. (1958) Proc. Roy. Soc. (London) B149:354.

Goldberg, A.L. (1969a) J. Biol. Chem. 244:3217.

Goldberg, A.L. (1969b) J. Biol. Chem. 244:3223.

Goldberg, A.L., and Chang, T.W. (1978) Fed. Proc. 37:2301.

Goldberg, A.L., and Dice, J.F. (1974) Ann. Rev. Biochem. 43:835.

Goldberg, A.L., Howell, E.M., Li, J.B., Martel, S.B., and Prouty, W.F. (1974) Fed. Proc. 33:1112.

Goldberg, A.L., and Odessey, R. (1972) Am. J. Physiol. 223: 1384.

Goldberg, A.L., Olden, K., and Prouty, W.F. (1975) In "Intracellular Protein Turnover" (Schimke, R.T., and Katunuma, N., eds.) p. 17, Acad. Press, New York.

Goldberger, J., and Little, R.D. (1926) U.S. Pub. Health Rep. 41:201

Goldberger, J., and Tanner, W.F. (1925) U.S. Pub. Health Rep. 40:58.

Goldberger, J., Wheeler, G.A., and Sydenstricker, V.P. (1918) J. Am. Med. Assoc. 71:944.

Goldberger, J., and Wheeler, G.A. (1928) U.S. Pub. Health Rep. 43:172.

Goldman, D.S. (1954) J. Am. Chem. Soc. 76:2841.

Gopalan, C., and Srinkantia, S.G. (1960) Lancet. 1:954.

Goswami, M.N.D., and Chatagner, F. (1966) Experientia. 22:1.

Greenbaum, A.L., and Pinder,S. (1968) Biochem. J. 107:55.

Greengard, P., Quinn, G.P., amd Reid, M.B. (1964) J. Biol. Chem. 239 :1887.

Greengard, P., Kalinsky,H., Manning, T.J., and Zak, S.B. (1968) J. Biol. Chem. 243:624.

Griffin, G.E., and Goldberg, A.L. (1978) J. Biol. Chem. In Press.

Grisolia, S. (1964) Physiol. Rev. 44:657.

Grosche, A.C., and Briggs, G.M. (1946) J. Biol. Chem. 165: 741.

Grosche, A.C., Anderson, J.O., and Briggs, G.M. (1947) Fed. Proc. 6:257.

Groschke, A.C., Anderson, J.C., and Briggs, G.M. (1948) Proc. Soc. Exp. Biol. Med. 68:564

Grossman, A., and Mavrides, C. (1967) J. Biol. Chem. 242: 1398.

Grossowicz, N., and Halpern, Y.S. (1956) Nature. 177:623.

Grunicke, H., Liersch, M., Hinz, M., and Holzer, H. (1966) Biochim. Biophys. Acta. 121:228.

Guggenheim, K., and Diamant, E.J. (1958) Biochem. J. 69:56.

Gurd, J.W., and Evans, W.H. (1973) Eur. J. Biochem. 36:273.

Gutman, A., and Shafrir, E. (1964) Proc. Soc. Exp. Biol. 117:264.

Hafez, Y.S., and Allred, J.B. (1978) J. Carbohydrates. Nucleosides. Nucleotides. 5:59.

Hagino, Y., Lan, S.J., Ng, C.Y., and Henderson, L.M. (1968) J. Biol. Chem. 213:4980.

Hainer, V., Kubik, V., Krulich, L., and Stoilov, S. (1972) Horm. Metab. Res. 4:314.

Haines, M.E., Johnston, I.R., Mathias, A.P., and Ridge, D. (1969) Biochem. J. 115:881.

Halliday, S.L., Sloboda, A., Will, L.W., and Olson, J.J. (1957) Fed. Proc. 16:190.

Handler, O., and Dann, W.J. (1942) J. Biol. Chem. 146:357.

Hankes, L.L., Henderson, L.M., Brickson, W.L., and Elvehjem, C.A. (1948) J. Biol. Chem. 174:873.

Hankes, L.L., Henderson, L.M., and Elvehjem, C.A. (1949) J. Biol. Chem. 180:1027.

Harper, A.E. (1959) Biochem. J. 71:702.

Harper, A.E., Benton, D.A., and Elvehjem, C.A. (1955) Arch. Biochem. Biophys. 57:1.

Harper, A.E., Benton, D.A., Winje, M.E., and Elvehjem, C.A. (1954) Arch. Biochem. Biophys. 51:523.

Haskins, F.A., and Mitchell, H.K. (1949) Proc. Natl. Acad. Sci. U.S.A. 35:500.

Hayaishi, O., Ijichi, H., and Ichiyama, A. (1966) J. Biol. Chem. 241:3701.

Hayashi, M., Hiroi, Y., and Natari, Y. (1973) Nature. New. Biol. 242:163.

Heidelberger, C., Araham, E.P., and Lepkovsky, S. (1949) J. Biol. Chem. 179:151.

Heidelberger, C., Gullberg, M.E., and Lepkovsky, S. (1949) J. Biol. Chem. 179:143.

Heinz, F., and Kulbe, K.D. (1971) Hoppe-Seyer's Z. Physiol. Chem. 351:249.

Helmer, O.M., and Fouts, P.J. (1938) J. Nutr. 16:271.

Henderson, A.R. (1970) Biochem. J. 120:205.

Henderson, L.M., Deodhar, T., Krehl, W.A., and Elvehjem, C.A. (1947) J. Biol. Chem. 170:261.

Henderson, L.M., and Gross, C.J. (1979) J. Nutr. 109:646.

Henderson, L.M., and Hankes, L.V. (1956) J. Biol. Chem. 222: 1069.

Henderson, L.M., Koeppe, O.J., and Zimmerman, H.H. (1953a) J. Biol. Chem. 201:529.

Henderson, L.M., Koeppe, O.J., and Zimmerman, H.H. (1953b) J. Biol. Chem. 201:697.

Henshaw, E.C., Hirsch, C.A., Morton, B.E., and Hiatt, H.H. (1971) J. Biol. Chem. 246:436.

Herken, H., and Lange, K. (1969) Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak. 263:496.

Herken, H., and Neuhoff, V. (1964) Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak. 247:187.

Hershko, A., Mamont, P., Shields, R., and Tomkins, G.M. (1971) Nature. New. Biol. 232:206.

Hershko, A., and Tomkins, G.M. (1971) J. Biol. Chem. 246: 710.

Hicks, S.P. (1955) Am. J. Pathol. 31:189.

Hill, A.B. (1961) Principles of Medical Statistics, The Lancet Limited, London, p. 146.

Hilz, H., and Stone, P. (1976) Rev. Physiol. Biochem. Pharmacol. 76:1.

Hirota, Y., Cohen, E.M., and Bing, O.H.L. (1976) Metab. 25:1.

Hirsch-Kolb, H., and Greenberg, D.M. (1969) J. Biol. Chem. 243:6123.

Holten, D., and Kenney, F.T. (1967) J. Biol. Chem. 242: 4372.

Holzer, H., Busch, D., and Kröger, H. (1958) Z. Physiol. Chem. 313:181.

Honjo, T., Nishizuka, Y., Kato, I., and Hayaishi, O. (1971) J. Biol. Chem. 246:4251.

Hopgood, M.F., and Ballard, F.J. (1974) Biochem. J. 144: 371.

Hopper, J.H., and Johnson, B.C. (1955) J. Nutr. 56:303.

Horecker, B.L., and Kornberg, A. (1948) J. Biol. Chem. 175:385.

Hsu, R.Y., and Lardy, H.A. (1969) In "Methods in Enzymology" (Lowenstein, J.M., ed.) Vol. XIII, p. 230, Acad. Press, New York.

Huber, C. (1867) Annalen. 141:271.

Huff, J.W., and Perlzweig, W.A. (1941) J. Biol. Chem. 142: 401.

Hughes, P.A.M., and Raine, D.N. (1966) Clin. Chim. Acta. 14:399.

Hughes, D.E., and Williamson, D.H. (1953) Biochem. J. 55: 851.

Huisman, W., Bouma, J.M.W., and Gruber, M. (1974a) Nature. 240:428.

Huisman, W., Lanting, L., Bouma, J.M.W., and Gruber, M. (1974b) FEBS Lett. 45:129.

Hundley, J.M. (1947) J. Nutr. 34:253.

Hundley, J.M. (1949a) J. Biol. Chem. 181:1.

Hundley, J.M. (1949b) Proc. Soc. Exp. Biol. Med. 70:592.

Hundley, J.M. (1954) In "The Vitamins" (Sebrell, W.H., and Harris, R.S., eds.) p. 452, Acad. Press, New York.

Hunter, J.E., and Harper, A.E. (1976) J. Nutr. 106:653.

Hurt, W.W., Scheer, B.T., and Devel, H.J. (1949) Arch. Biochem. 21:37.

Huszar, G., and Elzinger, M. (1971) Biochem. 10:229.

Ichiyama, A., Nakamura, S., and Nishizuka, Y. (1967) Arzneimittel-Forsch. 17:1346.

Ijichi, H., Ichiyama, A., and Hayaishi, O. (1966) J. Biol. Chem. 241:3701.

Ikeda, M., Tsuji, H., Nakamura, S., Ichiyama, A., Nishizuka, Y., and Hayaishi, O. (1965) J. Biol. Chem. 240:1395.

Inoue, H., and Pitot, H.C. (1970) Adv. Enz. Regul. 8:289.

Jacques, P.J. (1969) In "Lysosomes in Biology and Pathology" (Dingle, J.T., and Fells, H.B., eds.) Vol. 2, p. 395, North-Holland Publ., Amsterdam.

Jacobson, K.B., and Kaplan, N.O. (1957) J. Biol. Chem. 226: 603.

Jaenicke, R. (1969) In "Pyridine Nucleotide dependent Dehydrogenases" (Sunde, H., ed.) p. 69, Spring-Verlag.

Jaenicke, R. (1974) Eur. J. Biochem. 46:149.

Jandorf, B.J. (1943) J. Biol. Chem. 150:89.

Jaworek, D., Gruber, W., and Bergmeyer, H.U. (1974) In "Methods of Enzymatic Analysis" (Bergmeyer, H.U. ed.) Vol. 4, p. 2097, Verlag Chemie Weinheim, Acad. Press, New York.

Jedeikin, L.A., and Weinhouse, S. (1955) J. Biol. Chem. 213:271.

Johnson, R.W., and Kenney, F.T. (1973) J. Biol. Chem. 248: 4528.

Johnson, W.J., and McColl, J.D. (1955) Science. 122:834.

Johnson, W.J., and McColl, J.D. (1956) Fed. Proc. 15:284.

Jones, K.M. (1959) J. Biol. Chem. 73:714.

Jörnvall, H. (1975) J. Theor. Biol. 55:1.

Kallio, R.E., and Berg, C.P. (1949) J. Biol. Chem. 181:333.

Kaplan, N.O. (1959) Ciba Found. Study Group. 2:37.

Kaplan, N.O. (1960) In "The Enzymes" (Boyer, P.D., Lardy, H., and Myrbäck, K., eds.) Vol. III, p. 105, Acad. Press, New York.

Kaplan, N.O. (1961) In "Metabolic Pathways" (Greenberg, D.M., ed.) Vol. 2, p. 627, Acad. Press, New York.

Kaplan, N.O. (1966) In "Current Aspects of Biochemical Energenetics" (Kaplan, N.O., and Kennedy, E.P., eds.) p. 447, Acad. Press, New York.

Kaplan, N.O. (1968) J. Vitaminol. 14:103.

Kaplan, N.O., and Ciotti, M.M. (1954) J. Biol. Chem. 211: 431.

Kaplan, N.O., Ciotti, M.M., Stolzenbach, F.E., and Bacur, N.R. (1955) J. Am. Chem. Soc. 77:815.

Kaplan, N.O., Ciotti, M.M., and Stolzenbach, E.E. (1956) J. Biol. Chem. 221:833.

Kaplan, N.O., Colowick, S.P., and Barnes, C.C. (1951) J. Biol. Chem. 191:461.

Kaplan, N.O., Colowick, S.P., and Neufeld, E.F. (1953) J. Biol. Chem. 205:1.

Kaplan, N.O., Goldin, A., Humphreys, S.R., Ciotti, M.M., and Venditti, J.M. (1954) Science. 120:437.

Kaplan, N.O., Goldin, A., Humphreys, S.R., Ciotti, M.M., and Stolzenbach, F.E. (1956) J. Biol. Chem. 219: 287.

Karrer, P., and Manz, W. (1946) Helv. Chim. Acta. 29:1152.

Katunuma, N. (1973) Curr. Top. Cell. Regul. 7:37.

Katunuma, N., Kito, K., and Kominami, E. (1971) Biochem. Biophys. Res. Commun. 45:76.

Kauffman, F.C., and Johnson, E.C. (1974) J. Neurol. 5:379.

Kaufman, S. (1957) J. Biol. Chem. 226:511.

Kayne, H.L., Taylor, N., and Alpert, N.R. (1963) Arch. Biochem. Biophys. 100:13.

Kehr, W., Halbhübner, K., Loos, D., and Herken, H. (1978) Naunyn-Schmiedebergs Arch. Pharmacol. 304:317.

Kelsay, J., Miller, L.T., and Linkswiler, H. (1968) J. Nutr. 94:27.

Kenney, F.T. (1962) J. Biol. Chem. 237:1610.

Kenney, F.T. (1967) Science. 156:525.

Kidwell, W.R., and Watts, R. (1974) Fed. Proc. 33:1413.

Kirk, R.E. (1968) In "Experimental Design: Procedures for the Behavior Sciences" p. 91, Brooks/Cole Publishing Co., Belmont, Ca.

Kirschner, K., Gallego, E., Schuster, I., and Goodall, D. (1971) J. Mol. Biol. 58:29.

Klingenberg, M. (1974) In "Methods of Enzymatic Analysis" (Bergmeyer, H.U., ed.) Vol. 4, p. 2045, Verlag Chemie Weinheim, Acad. Press, New York.

Klose, J. (1975) Humangenetik. 26:231.

Knowles, S.E., Gunn, J.M., Hanson, R.W., and Ballard, F.J. (1975) Biochem. J. 146:595.

Knox, W.E. (1955a) In "Methods in Enzymology" (Colowick, S.P., and Kaplan, N.O., eds.) Vol. II, p. 242, Acad. Press, New York.

Knox, W.E., and Auerbach, V.H. (1955b) J. Biol. Chem. 214: 307.

Knox, W.E., and Mehler, A.H. (1951) Science. 113:237.

Koeppe, R., and Hall, R. (1956) Biochim. Biophys. Acta. 22:544.

Kohn, H.I. (1938) Biochem. J. 32:2075.

Koike, M., Reed, L.J., and Carroll, W.R. (1963) J. Biol. Chem. 238:30.

Kornacker, M., and Lowenstein, J.M. (1963) Biochem. J. 89: 272.

Koshland, D.E., Jr., and Neet, K.E. (1968) Ann. Rev. Biochem. 37:359.

Krebs, E.G. (1955) In "Methods in Enzymology" (Colowick, S.P., and Kaplan, N.O., eds.) Vol. I, p. 407.

Krehl, W.A., Sarma, P.S., Teply, L.J., and Elvehjem, C.A. (1946) J. Nutr. 31:85.

Krehl, W.A., Teply, L.J., Sarma, P.S., and Elvehjem, C.A. (1945) Science. 101:489.

Krishnaswamy, K., and Bapurao, S. (1978) Brit. J. Nutr. 39:61.

Krishnaswamy, K., and Gopalan, C. (1971) Lancet. ii:1167.

Krishnaswamy, K., Rao, S.B., Raghuram, T.C., and Srikantia, S.G. (1976) Am. J. Clin. Nutr. 29:177.

Kuehl, L., and Sumison, E.N. (1970) J. Biol. Chem. 245:6616.

Kutsky, R.K. (1973) In "Handbook of Vitamins and Hormones" van Nostrand Reinhold Co., p. 96.

Lane, R.S. (1967) Nature. 214:1331.

Lange, K., Kolbe, H., and Herken, H. (1970) Z. Physiol. Chem. 351:1241.

Lee, K.L., Darke, P.L., and Kenney, F.T. (1977) J. Biol. Chem. 252:4958.

Lee, K.H., and Kim, H.K. (1978) J. Biol. Chem. 253:8157.

Lee, K.L., Reel, J.R., and Kenney, F.T. (1970) J. Biol. Chem. 245:5806.

Lehninger, A.L. (1975) Biochemistry, 2nd ed., Worth Publishers, Inc., New York.

Leifer, E., Roth, L.J., Hogness, D.S., and Carson, M.H. (1951) J. Biol. Chem. 90:595.

Leveille, G.A. (1969) J. Nutr. 98:367.

Levy, H.R., Raineri, R.R., and Nevaldine, B.H. (1966) J. Biol. Chem. 241:2181.

Li, J.B., and Goldberg, A.L. (1976) Am. J. Physiol. In Press.

Li, J.B., Higgins, J.E., and Jefferson, L.S. (1979) Am. J. Physiol. 5:E222.

Li, J.B., and Knox, W.E. (1972) J. Biol. Chem. 247:7550.

Lindstedt, S., and Prockop, D.J. (1961) J. Biol. Chem. 236: 1399.

Lison, H. (1970) Naunyn-Schmiedebergs Arch. Pharmak. 267: 155.

Litwack, G., and Rosenfield, S. (1973) Biochem. Biophys. Res. Commun. 52:181.

Loeb, J.N. (1970) Arch. Biochem. 139:306.

Long, C.L., Hill, H.N., Weinstock, I.M., and Henderson, L.M. (1954) J. Biol. Chem. 211:405.

Lorimer, W.S. III., Stone, P.R., and Kidwell, W.R. (1976) Fed. Proc. 35:1624

Lowry, O.H., Roberts, N.R., and Kapphahn, J.L. (1957) J. Biol. Chem. 224:1047.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193:265.

Majerus, P.W., Alberts, A.W., and Vagelos, P.R. (1965) J. Biol. Chem. 240:618.

Mallette, L.E., Exton, J.H., and Park, C.R. (1969) J. Biol. Chem. 244:5724.

Manchester, K.L., Randle, P.J., and Young, F.G. (1959) J. Endocrinol. 18:395.

Mann, P.J.G., and Quastel, J.H. (1941) Biochem. J. 35:502.

Marangos, P.J., and Constantinides, S.M. (1974a) J. Biol. Chem. 249:951.

Marangos, P.J., and Constantinides, S.M. (1974b) Biochem. 13:904.

Marks, P.A., Szeinberg, A., and Banks, J. (1961) J. Biol. Chem. 236:10.

Marquardt, R.R. (1969) Can. J. Biochem. 47:517.

Marshall, E.K., Bratton, A.C., White, H.J., and Litchfield, J.T. (1940) Johns. Hopkins. Hosp. Bull. 67:163.

Marzari (1817) Aleno de Treniso I.

Mason, M., and Berg, C.P. (1951) J. Biol. Chem. 188:783.

Mason, J.A., and Carpenter, K.J. (1978) J. Nutr. 109:1883.

Massey, V.C. (1960) Biochim. Biophys. Acta. 38:447.

Mauk, M.R., Putz, G.R., and Taketa, F. (1976) Biochem. Biophys. Res. Commun. 71:768.

McComb, R., and Gay, J. (1968) Clin. Chem. 14:754.

McDaniel, E.G., Hundley, J.M., and Sebrell, W.H. (1955) J. Nutr. 55:623.

McIlwain, H. (1940) Br. J. Exp. Pathol. 21:136.

McIlwain, H. (1950) Biochem. J. 46:612.

McLean, P. (1958) Biochim. Biophys. Acta. 30:316.

Mego, J.L., Bertini, F., and McQueen, J.O. (1967) J. Cell. Biol. 32:699.

Mego, J.L., Farb, R.M., and Barnes, J. (1972) Biochem. J. 128:763.

Mehler, A.H., and May, E.L. (1956) J. Biol. Chem. 223:449.

Meijer, A.J., Van Woerkom, G.M., Williamson, J.R., and Tager, J.M. (1975) Biochem. J. 150:205.

Mickelsen, O., Waisman, H.A., and Elvehjem, C.A. (1938) J. Biol. Chem. 124:313.

Miller, L.L. (1960) Nature. 185:248.

Miller, L.L. (1965) Fed. Proc. 24:737.

Millward, D.J. (1970) Clin. Sci. 39:591.

Millward, D.J., Garlick, P.J., James, W.P.T., Nnanynelugo, D.O., and Ryatt, J.S. (1973) Nature. 241:204.

Millward, D.J., Nnanynelugo, D.O., James, W.P.T., and Garlick, P.J. (1974) Br. J. Nutr. 33:127.

Minemura, T., Lacy, W.W., and Crofford, O.B. (1970) J. Biol. Chem. 245:3872.

Mitchell, H.K., and Nyc, J.F. (1948) Proc. Natl. Acad. Sci. U.S.A. 34:1.

Miyake, A., Bokman, A.H., and Schweigert, B.S. (1954) J. Biol. Chem. 211:391.

Momany, F.A., Aguanno, J.J., and Larrabee, A.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73:3093.

Morrison, M.A., Reynolds, M.S., and Harper, A.E. (1960) J. Nutr. 72:302.

Mortimore, G.E., and Mondon, C.E. (1970) J. Biol. Chem. 245:2375.

Moss, J., Manganiello, V.C., and Vaughan, M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73:4424.

Munro, H.N. (1964) In "Mammalian Protein Metabolism" (Munro, H.N., ed.) Vol. I, p. 382, Acad. Press, New York.

Murty, C.N., Verney, E., and Sidransky, H. (1976) Lab. Invest. 34:77.

Murty, C.N., Verney, E., and Sidransky, H. (1977) Biochim. Biophys. Acta. 474:117.

Najjar, V.A., and Holt, L.E. (1941) Science. 93:20.

Nakagawa, I., Ohguri, S., Sasaki, A., Kajimoto, M., Sasaki, M., and Takahashi, T. (1975) J. Nutr. 105:1241.

Nakagawa, I., and Sasaki, A. (1977) J. Nutr. Sci. Vitaminol. 23:535.

Neeley, A.N., Nelson, P.B., and Mortimore, G.E. (1974) Biochim. Biophys. Acta. 338:458.

Nelson, T.S., and Scott, H.M. (1953) Poult. Sci. 32:601.

Nettleton, J.A., and Hegsted, D.M. (1974) J. Nutr. 104:916.

Neubert, D., Schulz, H.U., and Hoehne, R. (1964) Biochim. Biophys. Acta. 92:610.

Neuhoff, V., and Desselberger, E. (1965) Naunyn-Schmiedebergs Arch. Exp. Path. Pharmakol. 252:43.

Newsholme, E.A., and Start, C. (1973) "Regulation in Metabolism", p. 290, John Wiley and Sons, New York.

Nirenberg, M.W. (1961) Proc. Natl. Acad. Sci. U.S.A. 47: 1588.

Nishizawa, N., Shimbo, M., Hareyama, S., and Funabiki, R. (1977) Br. J. Nutr. 37:345.

Nishizuka, Y., and Hayaishi, O. (1963a) J. Biol. Chem. 238:483.

Nishizuka, Y., and Hayaishi, O. (1963b) J. Biol. Chem. 238: 3369.

Nishizuka, Y., Ueda, K., and Hayaishi, O. (1971) In "Methods in Enzymology" (McCormick, D.B., and Wright, L.D., eds.) Vol. 18B, p. 230, Acad. Press, New York.

Nishizuka, Y., Ueda, K., Nakazawa, K., and Hayaishi, O. (1967) J. Biol. Chem. 242:3164.

Nordie, R.C., Varricchio, F.E., and Holten, D.D. (1965) Biochim. Biophys. Acta. 97:214.

Ogata, E.S., Foung, S.K.H., and Holliday, M.A. (1978) J. Nutr. 108:759.

Ohtsu, E., Ichiyama, A., Nishizuka, Y., and Hayaishi, O. (1967) Biochem. Biophys. Res. Commun. 29:635.

Olivera, V.B.M., and Lehman, I.R. (1967) Proc. Natl. Acad. Sci. 57:1700.

Olson, J.P., and Marquardt, R.R. (1972) Biochim. Biophys. Acta. 268:453.

Ovadi, J., Telegdi, M., Batke, J., and Keleti, T. (1971) Eur. J. Biochem. 22:430.

Pace, C.N. (1975) Crit. Rev. Biochem. 1:1.

Pande, S.V., Parvin, K.R., and Venkitasubramanian, T.A. (1964) Biochim. Biophys. Acta. 84:239.

Pande, S.V., Parvin, K.R., and Venkitasubramanian, T.A. (1965) Biochim. Biophys. Acta. 100:344.

Pappenheimer, A.M. (1977) Ann. Rev. Biochem. 46:69.

Parr, C.W. (1956) Nature. 178:1401.

Partridge, C.W.H., Bonner, D.H., and Yanofsky, C. (1952) J. Biol. Chem. 194:269.

Pearson, W.N., and Song, C.S. (1963) "In Nutrition, Proceedings of the 6th International Congress", Edinburgh, p. 482 (Abst.).

Penny, D., Anderson, D., and Dongas, J. (1976) J. Nutr. 106:1235.

Petrack, B., Greengard, P., Craston, A., and Kalinsky, J. (1963) Biochem. Biophys. Res. Commun. 13:474.

Pirias, M., and Knox, W.E. (1967) J. Biol. Chem. 242:2959.

Polakis, E.S., and Bartley, W. (1966) Biochem. J. 99:521.

Pontremoli, S., and Grazi, E. (1966) In "Methods in Enzymology" (Wood, W.A., ed.) Vol. IX, p. 137.

Pontremoli, S., Melloni, E., De Flora, A., and Horecker, B.L. (1973) Proc. Natl. Acad. Sci. U.S.A. 70:3674.

Poole, B., and Wibo, M. (1973) J. Biol. Chem. 248:6221.

Powanda, M.C., Muniz, O., and Dietrich, L.S. (1969) Biochem. 8:1869.

Powick, W.C., Ellis, N.R., Madsen, L.L., and Dale, C.N. (1947) J. Anim. Sci. 6:310.

Preiss, J., and Handler, P. (1957) J. Biol. Chem. 150:447.

Preiss, J., and Handler, P. (1958) J. Biol. Chem. 233:493.

Priest, R.E., Bokman, A.H., and Schweigert, B.S. (1951) Proc. Soc. Exp. Biol. Med. 78:477.

Pullman, M.E., and Colowick, S.P. (1954a) J. Biol. Chem. 206:121.

Pullman, M.E., San Pietro, A., and Colowick, S.P. (1954b) J. Biol. Chem. 206:129.

Quastel, J.H., and Zatman, L.J. (1953) Biochim. Biophys. Acta. 10:256.

Rabinowitz, M., and Fisher, J.M. (1961) Biochem. Biophys. Res. Commun. 6:449.

Raghuramulu, N., Rao, N.B.S., and Gopalan, C. (1965a) J. Nutr. 86:100.

Raghuramulu, N., Srikantia, S.G., Narasinga Rao, B.S., and Gopalan, C. (1965b) Biochem. J. 96:837.

Randle, P.J., Garland, P.B., Hales, C.N., Newsholme, E.A., Denton, R.M., and Pogson, C.J. (1966) Recent. Prog. Horm. Res. 22:1.

Rao, N.B.S., and Ghafoorunissa. (1972) Am. J. Clin. Nutr. 25:6.

Rechsteiner, M., Hillyard, D., and Oliver, B.M. (1976) Nature. 259:695.

Reddi, K.K., and Kodicek, E. (1953) Biochem. J. 53:286.

Regen, D., Riepertinger, C., Hamprecht, B., and Lynn, F. (1966) Biochem. Z. 346:78.

Rickwood, D., and Klemperer, H.G. (1971) Biochem. J. 123: 731.

Roberts, J.H., Stark, P., and Smulson, M.E. (1973) Biochem. Biophys. Res. Commun. 52:43.

Roberts, J.H., Stark, P., and Smulson, M.E. (1974) Proc. Natl. Acad. Sci. U.S.A. 71:3212.

Roberts, R.M., and Yuan, B.O.C. (1975) Arch. Biochem. Biophys. 171:226.

Robinson, F.A. (1966) The Vitamin Cofactors of Enzyme Systems, 1st ed., p. 274, Permagon Press Ltd.

Rodkey, F.L. (1955) J. Biol. Chem. 213:777.

Rogers, Q.R., Tannous, R.I., and Harper, A.E. (1967) J. Nutr. 91:561.

Rognstad, R., and Katz, J. (1976) Arch. Biochem. Biophys. 177:337.

Rose, D.P., and McGinty, F. (1970) Adv. Steroid. Biochem. Pharmacol. 1:97.

Rose, D.P., and Toseland, R.A. (1973) Metab. 22:165.

Rosen, F., and Perlzweig, W.A. (1949) J. Biol. Chem. 177: 163.

Rosenberg, H.R. (1945) In "Chemistry and Physiology of the Vitamin", p. 219, Interscience Publishers, Inc., New York.

Rottman, F., Shatkin, A.J., and Perry, R.P. (1974) Cell. 3:197.

Rudolph, R., Heider, I., Westhof, E., and Jaenicke, R. (1977) Biochem. 16:3384.

Rutter, W.J., Rajkumar, T., Penhoet, E., Kochman, M., and Valentine, R. (1968) Ann. N. Y. Acad. Sci. 151:102.

Sakurai, T., Miyazawa, S., and Hashimoto, T. (1973) FEBS Lett. 36:96.

Sarett, H.P. (1942) J. Nutr. 23:35.

Satyanarayana, U., and Rao, B.S.N. (1977) J. Nutr. 107:2213.

Schaefer, A.E., McKibbin, J.M., and Elvehjem, C.A. (1942) J. Biol. Chem. 144:679.

Schayer, R.W., Foster, G.L., and Shemin, D. (1949) Fed. Proc. 8:248.

Schimke, R.T. (1964) J. Biol. Chem. 239:3808.

Schimke, R.T., Sweeney, E.W., and Berlin, C.M. (1965) J. Biol. Chem. 240:322.

Schlenk, F. (1945) In "Methoden der Fermentforschung" (Bamann, E., and Myrbäck, K., eds.) Acad. Press, New York.

Schlenk, F. (1951) In "The Enzymes" (Sumner, J.B., and Myrbäck, K., eds.) Vol. II, p. 250, Acad. Press, New York.

Scott, M.L., Holm, E.R., and Reynolds, R.E. (1959) Poult. Sci. 38:1344.

Scott, M.L., Nesheim, M.C., and Young, R.T. (1976) In "Nutrition of the Chicken", 2nd. ed., p. 208, M.L. Scott and Associates Publishers, New York.

Scrutton, M.L., and Utter, M.F. (1968) Ann. Rev. Biochem. 37:249.

Sedgewick, K.A., and Frieden, C. (1968) Biochem. Biophys. Res. Commun. 32:392.

Segal, H.L., Winkler, J.R., and Miyagi, M.P. (1974) J. Biol. Chem. 249:6364.

Seitz, H.J., Kaiser, M., Krone, W., and Tarnowski, W. (1976) Metab. 25:1545.

Shapiro, D.M., Dietrich, L.S., and Shils, M.E. (1957) Cancer. Res. 17:600.

Shimizu, C.S.N., and Kaplan, S.A. (1964) Endocrinol. 74:709.

Siekevitz, P. (1952) J. Biol. Chem. 195:549.

Silpananta, P., and Goodridge, A.G. (1971) J. Biol. Chem. 246:5754.

Simpson, M.V. (1953) J. Biol. Chem. 201:143.

Singal, S.A., Sydenstricker, V.P., and Littlejohn, J.M. (1947) J. Biol. Chem. 171:203.

Singal, S.A., Sydenstricker, V.P., and Littlejohn, J.M. (1948a) J. Biol. Chem. 176:1051.

Singal, S.A., Sydenstricker, V.P., and Littlejohn, J.M. (1948b) J. Biol. Chem. 176:1069.

Smulson, M., Giri, Ch., Sudhakar, S., and Mullins, D. (1976) In Proc. 4th Int. Symp. Poly (ADP-ribose) and ADP-ribosylation of Proteins (Hilz, H., ed.), p. 7.

Snedecor, G.W. (1956) Statistical Methods 5th ed. Iowa State Univ. Press, Ames, Iowa, p. 329.

Snedecor, G.W., and Cochran, W.G. (1967) Statistical Methods 6th ed. Iowa State Univ. Press, Ames, Iowa, p. 349.

Snell, E.E., and Quarles, E. (1941) J. Nutr. 22:483.

Snyderman, S.E., Ketron, K.C., and Holt, L.E. (1949) Proc. Soc. Exp. Biol. Med. 70:569.

Solomon, G., and Tarver, H. (1952) J. Biol. Chem. 195:447.

Spies, T.D., Cooper, C., and Blankenhorn, M.A. (1938) J. Am. Med. Assoc. 110:622.

Spirtes, M.A., and Alper, C. (1961) J. Nutr. 73:374.

Srikantia, S.G., Reddy, V., and Krishnaswamy, K. (1968) Neurophysiol. 25:386.

Stanley, B.G., Neal, G.E., and Williams, D.C. (1971) In "Methods in Enzymology" (McCormick, D.B., and Wright, L.D., eds.) Vol. XVIII, Part B, p. 775.

Sternberg, S.S., and Philips, F.S. (1958) Science. 127:644.

Stirewalt, W.S., Wool, I.G., and Cavicchi, T. (1968) Proc. Natl. Acad. Sci. 57:1885.

Stockell, A. (1959) J. Biol. Chem. 234:1293.

Streffer, C., and Benes, J. (1971) Eur. J. Biochem. 21:357.

Subramanian, S., and Ross, P.D. (1977) Biochem. Biophys. Res. Commun. 78:461.

Subramanian, S., and Ross, P.D. (1978) Biochem. 17:2193.

Sunde, M.L., and Bird, H.R. (1957) Poult. Sci. 36:34.

Sure, B., Easterling, L., and Jen, S. (1952) J. Nutr. 46:55.

Suzuki, U., and Matsunaga, S. (1912) J. Agr. Tokyo. Imp. Univ. 5:59.

Suzuki, U., Shamimura, T., and Okade, S. (1912) Biochem. Z. 43:89.

Svedmyr, N., and Harthon, L. (1970) Acta. Pharmacol. Toxicol. 28:66.

Sydenstricker, V.P. (1958) Am. J. Clin. Nutr. 6:409.

Taguchi, H., and Iwai, K. (1974) J. Nutr. Sci. Vitamin. 20:283.

Takenaka, Y., and Schwert, G.W. (1956) J. Biol. Chem. 223: 157.

Tannous, R.I., Rogers, Q.R., and Harper, A.E. (1966) Arch. Biochem. Biophys. 113:356.

Tappan, D.V., Lewis, U.J., Register, U.D., and Elvehjem, C.A. (1952) J. Nutr. 46:75.

Taylor, J.F., Velick, S.F., Cori, G.T., and Slein, M.W. (1948) J. Biol. Chem. 173:619.

Teply, L.J., Krehl, W.A., and Elvehjem, C.A. (1947) Am. J. Physiol. 148:91.

Theorell, H. (1934) Biochem. Z. 275:11.

Truswell, A.S., Goldsmith, G.A., and Pearson, W.N. (1963) Lancet. 1:778.

Tupule, P.G. (1958) Ind. J. Med. Res. 46:706.

Tuttle, W.L., and Balloun, S.L. (1976) Poult. Sci. 55:1737.

Tweto, J., Dehlinger, P.J., and Larrabee, A.R. (1972) Biochem. Biophys. Res. Commun. 48:1371.

Utter, M.F. (1959) Ann. N. Y. Acad. Sci. 72:451.

Valeriote, F.A., Auricchio, F., Tomkins, G.M., and Riley, D. (1969) J. Biol. Chem. 244:3618.

Van Eys, J., Ciotti, M.M., and Kaplan, N.O. (1958) J. Biol. Chem. 231:571.

Velick, S.F. (1953) J. Biol. Chem. 203:563.

Velick, S.F. (1955) In "Methods in Enzymology" (Colowick, S.P., and Kaplan, N.O., eds.) Vol. I, p. 401, Acad. Press, New York.

Veneziale, C.M., Walter, P., Kneer, N., and Lardy, H.A. (1967) Biochem. 6:2129.

Vennesland, B., and Westheimer, F.H. (1954) In "The Mechanisms of Enzyme Action" (McElroy, W.D., and Glass, B., eds.) Johns Hopkins, Baltimore.

Vesell, E.S., and Yielding, K.L. (1966) Proc. Natl. Acad. Sci. U.S.A. 56:1311.

Vilter, R.W., Vilter, S.P., and Spies, T.D. (1939) J. Am. Med. Assn. 112:420.

von Euler, H. (1942) Ber. 75:1876.

von Euler, H., Adler, E., and Hellström, H. (1936) Z. Physiol. Chem. Hoppe-Seyler's. 240:239.

von Euler, H., and Heiwinkel, H. (1937) Naturwissenschaften. 25:269.

von Euler, H., Schlenk, F., and Högoerg, B. (1938) Z. Physiol. Chem. 256:208.

Wang, T.P., and Kaplan, N.O. (1954) J. Biol. Chem. 206:311.

Warburg, O., and Christian, W. (1935) Biochem. Z. 275:112.

Wassner, S.J., Orloff, S., and Holliday, M.A. (1977) Am. J. Physiol. 233:E119.

Waterlow, J.C., and Stephen, J.M.L. (1968) Clin. Sci. 35: 287.

Webb, J.L. (1966) Enzyme and Metabolic Inhibitors, Vol. II, p. 482, Acad. Press, New York.

Weber, G., and Canters, A. (1957) Am. J. Physiol. 190:229.

Weiner, M. (1979) Drug. Metab. Rev. 9:99.

Wildenthal, K. (1973) Nature. 243:226.

Wilkening, M.C., and Schweigert, B.S. (1947) J. Biol. Chem. 171:209.

Williams, J.N., Jr., Feigelson, P., and Elvehjem, C.A. (1950) J. Biol. Chem. 187:597.

Williamson, D.H., Mayor, F., and Veloso, D. (1971) In "Regulation of Gluconeogenesis" (Söling, H.D., and Willms, B., eds.) p. 92, Acad. Press, New York.

Wimhurst, J.M., and Manchester, K.L. (1973) Biochem. J. 134:143.

Wise, E.M., and Rall, E.G. (1964) Proc. Natl. Acad. Sci. U.S.A. 52:1255.

Wiseman, A., and Williams, N.J. (1971) Biochim. Biophys. Acta. 250:1.

Wiss, O., and Weber, F. (1964) Vitamins and Hormones. 22: 495.

Wittman, J.S. III., Lee, K.L., and Miller, O.N. (1969) Biochim. Biophys. Acta. 174:536.

Wollenberger, A., Ristau, O., and Schaffa, G. (1960) Arch. Ges. Physiol., Pfluger's. 270:399.

Wool, I.G., and Weinshelbaum, E.I. (1960) Am. J. Physiol. 198:360.

Woolley, D.W. (1945) J. Biol. Chem. 157:455.

Woolley, D.W., Strong, F.M., Madden, T.J., and Elvehjem, C.A. (1938) J. Biol. Chem. 124:715.

Woolley, D.W., and White, A.G.C. (1943) Proc. Soc. Exp. Biol. Med. 52:106.

Wu, S.Y., and Rosenthal, H.L. (1966) Proc. Soc. Exp. Biol. Med. 122:414.

Wunner, W.H. (1967) Proc. Nutr. Soc. 27:153.

Wunner, W.H., Bell, J., and Munro, H.N. (1966) Biochem. J. 101:417.

Yonetani, T., and Theorell, H. (1962) Arch. Biochem. Biophys. 99:433.

Young, V.R., Alexis, S.D., Baliga, B.S., Munro, H.N., and Muecke, W. (1972) J. Biol. Chem. 247:3593.

Young, V.R., Haverberg, L.N., Bilmazes, C., and Munro, H.N. (1973) Metab. 22:1429.

Zaman, Z., and Verwilghen, R.L. (1975) Biochem. Biophys. Res. Commun. 67:1192.

Zatman, L.J., Kaplan, N.O., Colowick, S.P., and Ciotti, M.M. (1954a) J. Biol. Chem. 209:453.

Zeitz, M., Lange, K., Keller, K., and Herken, H. (1978) Naunyn-Schmiedeberg's Arch. Pharmacol. 305:117.

Zondag, H.A. (1963) Science. 142:965.

(1) Medicines (Extension to Antimicrobial Substances) Order 193. No. 367 : London : H.M.S.O. 1973.

Recommended Dietary Allowance (1974) 8th edn. Food and Nutrition Board, Nat. Acad. of Sci, Washington, D.C.