THE EFFECT OF PARATHYROID HORMONE ON RESPIRATION IN ISOLATED RAT LIVER CELLS

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by

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ABSTRACT

The Effect of Parathyroid Hormone on Respiration in Isolated Rat Liver Cells

The role of parathyroid hormone (PTH) in calcium and phosphate regulation in the body is well established but there is no generally accepted hypothesis for the action of the hormone at the cellular level. Since PTH appears to involve oxidative phosphorylation in mitochondrial systems in vitro, the purpose of the present work was to establish and examine the conditions under which an in vitro effect of PTH on cellular respiration rate could occur and to study the specificity of this hormonal effect.

Liver cells were obtained by differential centrifugation after mild homogenization of perfused rat liver. Hormone was isolated by the phenol extraction gel-filtration technique of Aurbach and Potts (Endocrinology 75, 290, 1964). The cell preparation was incubated in Warburg flasks containing the optimal concentrations of 300mM mannitol, 20mM sodium phosphate pH 7.2, 15mM sodium succinate pH 7.2 and 1mM $MgCl_2$. Oxygen uptake was determined by standard manometric techniques.

The control rate in the above basic medium was 60 $\mu l O_2/hr/10^6$ cells. Oligomycin (3.85μg/ml) or 0.5mM ATP, separately, had no effect on the respiration rate but together reduced it to about 30 $\mu l O_2/hr/10^6$ cells. PTH (8.5μg/ml) had no effect on the control respiration rate but completely

overcame the reduction in respiration which occurred after oligomycin and ATP addition. Oxidized or heated (100°C) hormone did not function in this system whereas oxidized hormone that had been reduced again did. UTP, CTP, GTP and ITP had the same effect in the system as oligomycin and ATP combined. ATP itself could be completely replaced by ADP or AMP and partially by adenosine but oligomycin was still required. In the absence of oligomycin, interconversion of adenosine nucleotide was shown to occur governed by adenylate kinase, ATPase and oxidative phosphorylation activities. The reduction in respiration by ITP was overcome if ATP, ADP or AMP was also added to the medium. Other basic proteins (protamine sulfate and polylysine) and uncouplers (gramicidin and dinitrophenol) were as effective as PTH in relieving inhibition by oligomycin and ATP. If magnesium or phosphate was omitted from the incubation medium the respiration rate was reduced to about 30 $\mu l O_2/hr/10^6$ cells. In phosphate deficient but not in magnesium deficient mediums, PTH returned the oxygen uptake to control values only if nucleotide phosphate was present. In this case the hormone could not be replaced by dinitrophenol, polylysine, oxidized PTH or heated (100°C) PTH. However, oxidized hormone that had been reduced again functioned.

Although there is no definitive evidence, it is probable that PTH penetrates the cells and acts directly upon the mitochondrion. Effects of PTH on mitochondrial respiration

has been interpreted in several ways in the past. These have included stimulation of ATPase activity, uncoupling of oxidative phosphorylation and direction of energy into ion translocation. In the present system it is believed that the nucleotide phosphates improve the functional integrity of the mitochondrion and that the hormone is not stimulating an ATPase but rather functioning as an uncoupler of oxidative phosphorylation. However, its action is not identical with that of dinitrophenol nor is the basic nature of the hormone the only factor involved. These complex effects on isolated cells are compared to studies on mitochondria and discussed in detail.

ABBREVIATIONS

The following abbreviations are used:

PTH, parathyroid hormone; ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; ITP, inosine triphosphate; IDP, inosine diphosphate; IMP, inosine monophosphate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; NAD, nicotinamide-adenine dinucleotide; NADH, reduced nicotinamide-adenine dinucleotide; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; FAD, flavin-adenine dinucleotide; DNP, dinitrophenol; $^{32}P_i$, radioactive inorganic phosphate; GSH, glutathione; CoA, coenzyme A; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetate; Tris buffer, tris (hydroxymethyl) amino methane; ^{o}C, degrees centigrade; mμ, millimicron; O.D., optical density; s.d., standard deviation; cm, centimetre; mm, millimetre; ml, millilitre; μl, microlitre; gr, gram; mg, milligram; μg, microgram; μμg, micromicrogram; N, normal; M, molar; mM, millimolar.

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INTRODUCTION

The physiological action of parathyroid hormone (PTH) in serum calcium and phosphate regulation has been known for many years. It has a direct effect on the mobilization of the skeleton, it stimulates calcium and phosphate uptake in the intestine and increases calcium reabsorption and phosphate excretion in the kidney. Although the liver is not an obvious target organ for PTH like the skeleton, intestine and kidney, there is considerable evidence that PTH does have a widespread action in the body. Various effects of PTH on mammary gland, lens, salivary gland, erythrocyte and muscle have been reported. More recently, effects of PTH at the subcellular level on liver and, to a lesser extent, on kidney mitochondria have been studied. However, there is no consensus of opinion as to how these physiological changes might be brought about at the cellular level.

The reasons for using rat liver cells in the present study were:

1) a suspension of isolated cells could be uniformly sampled and it appeared from the literature that the cells could be easily prepared in good yield from rat liver.
2) hormonal effects would be upon intact cells.
3) comparisons could be made with the recent work of others on liver mitochondria.

Briefly, the specific aims of the present study were:

1) to devise the optimal procedure for preparing isolated rat liver cells and a suitable medium in which to study cellular oxygen utilization.
2) to establish and examine the conditions under which an *in vitro* effect of PTH on cellular respiration rate could occur.
3) to study the specificity of this hormonal effect.

HISTORY OF THE LITERATURE

1) Parathyroid Hormone

General. Ever since the classic work of MacCallum and Voegtlin in 1909, it has been known that the parathyroid glands play an important role in controlling the concentrations of calcium and phosphate in the blood. However, a controversy existed concerning the mechanism of action of PTH. On the one hand, there were those investigators who favoured the view that the primary action of the hormone was upon renal excretion of phosphate and that changes in the concentration of calcium in the plasma and the subsequent mobilization of calcium from the bone were consequences of this renal effect. On the other hand, a number of investigators considered that the primary effect of PTH was upon resorption of the bone, the renal and other effects being secondary. The finding that parathyroidectomy resulted in a fall in the serum calcium of nephrectomized rats (Talmage et al, 1953) and the demonstration of a direct action of PTH on bone (Barnicot, 1948) and on kidney (Rasmussen and Deluca, 1963) made it obvious that neither of these diametrically opposed views was wholly correct.

Several studies have shown that calcium but not phosphate controls the secretion of PTH. Stoerk and Carnes (1945) demonstrated that parathyroid gland enlargement correlated almost perfectly with the serum calcium

concentration of the animals but showed no definite relation to the serum inorganic phosphate concentration. Roth et al (1964) have reported several effects of calcium on the ultrastructure of rat parathyroid glands. High calcium concentrations reduced the cell volume, size of the Golgi apparatus and the aggregation of ribonucleoprotein particles. These changes were correlated with a suppression of the liberation of ^{14}C-leucine labelled proteins. Low calcium concentrations had the opposite effects. More recently, the availability of sensitive radioimmunoassay has made possible direct determination of the level of circulating endogenous hormone. Melick et al (1965) have shown the biological half life to be relatively short, of the order of twenty-two minutes in the rat. Sherwood et al (1966) found the normal concentration range in bovine plasma to be between 400 and 1,800μμg/ml. They clearly established an inverse relationship existed between the concentration of PTH and of calcium but not phosphate. The plasma PTH concentration fell to less than 100μμg/ml when the plasma calcium was increased to 14mg % (4mg % above normal) by intravenous infusion of calcium chloride, and rose as high as 6,000μμg/ml by infusion of a solution of EDTA. The development of techniques for perfusion of isolated parathyroid glands in the sheep and goat permitted Care et al (1966) to measure the PTH concentration in the venous effluent of the glands. The rate of secretion of PTH was inversely proportional to, and responded rapidly

to changes in, the calcium concentration in the perfusate.

PTH is not the only hormone involved in the regulation of the blood serum calcium level. Thyrocalcitonin, which is secreted from the thyroid gland when the serum calcium level is high, has an effect opposed to that of PTH in reducing the calcium level (Munson, 1966). Thyrocalcitonin was suspected when surgical removal of the parathyroid glands resulted in a higher serum calcium level than occurred following destruction of the glands by electrocautery. It is probable that during electrocautery of the parathyroid glands the adjacent thyroid glands are directly stimulated to release thyrocalcitonin.

Potts et al (1965) have proposed a model structure for PTH consisting of a single chain polypeptide with a molecular weight of approximately 9,000. The amino acid composition is $lysine_{10}$, $histidine_4$, $arginine_5$, $aspartic\ acid_8$, $serine_6$, $glutamic\ acid_{10}$, $proline_3$, $glycine_4$, $valine_7$, $alanine_7$, $methionine_2$, $isoleucine_3$, $leucine_8$, $tyrosine_1$, $phenylalanine_2$ and $tryptophan_1$. Their partial analysis of amino acid sequence showed lysine and arginine to be clustered in certain areas giving these regions a highly positive charge. A minimum structure requisite for biological activity appeared to be approximately 25% of the molecule, a sequence of twenty amino acid residues at the carboxyl terminal of the polypeptide chain.

Oxidation of PTH by hydrogen peroxide or performic

acid resulted in the loss of both the calcium mobilizing and phosphaturic activities of the hormone in vivo (Tashjian, Ontjes and Munson, 1964). If the oxidized PTH was reduced at elevated temperatures with mercaptoethanol or cysteine hydrochloride, biological activity was again restored. They concluded that this activity was dependent on methionine because this was the sole amino acid residue found to undergo reversible oxidation-reduction. It is now known that oxidation of the tyrosine or tryptophan residues of PTH by N-bromosuccinimide in 8M urea also causes marked loss of biological activity (Potts et al, 1966). There was no loss of antigenic activity of the hormone following oxidation of methionine (Tashjian, Ontjes and Munson, 1964) or tryptophan (Aurbach and Potts, 1967) indicating that the sites of biological activity and immunological activity are not identical and that no major alteration in the conformity of the molecule occurred.

Effects of PTH on Bone. The first studies which clearly demonstrated a direct effect of PTH on bone were carried out by Barnicot (1948). He transplanted parathyroid tissue into direct contact with one surface of the bone of the skull. Bone resorption occurred on this surface and bone deposition on the opposite surface. The finding that PTH stimulates bone resorption has been verified by others (Chang, 1951 and Gaillard, 1959) but no generally accepted mechanism has yet been proposed. The resorption of bone is

a complex process in which the breakdown of ground substance and collagen fibers as well as the hydroxyapatite crystals occurs (Rasmussen, 1961).

Changes in carbohydrate metabolism may be involved. Neuman and Neuman (1957) suggested that the stimulation of citric and lactic acid production by PTH may be responsible for the mobilization of bone. The solubility of hydroxyapatite in the extracellular fluid would increase due to the increased citrate ion concentration and also due to the decrease in pH caused by an increase in lactate concentration. This suggestion by Neuman and Neuman was supported by other workers. Firschein et al (1958) found that following the injection of parathyroid extract into dogs, the serum citrate rose before the serum calcium. Martin et al (1964) reported that parathyroid extract increased the utilization of glucose and the production of lactate and citrate by calvaria in tissue culture. They proposed that the increase in citrate was due to a reduced isocitric dehydrogenase activity. On the other hand, Cohn et al (1965) found that tricarboxylic acid intermediates did not accumulate during <u>in vitro</u> incubation of bone slices prepared from rabbits injected with parathyroid extract despite a large inhibition of tricarboxylic acid cycle oxidation. Dowse et al (1963), adding PTH to rat calvaria incubated <u>in vitro</u>, also found no significant changes in citrate accumulation. The only significant finding was an increased production of lactate

which only occurred under aerobic conditions. Obviously, more data are required before the role of citrate and lactate in this complex process are understood.

Effects of PTH on bone probably also involve protein synthesis. Nichols et al (1965) showed that incorporation of ^{14}C-labelled amino acids into collagen of isolated mouse bone cells was depressed following injection of parathyroid extract. Rasmussen, Arnaud and Hawker (1964) and Tashjian, Ontjes and Goodfriend (1964) demonstrated that actinomycin D, a compound that blocks DNA-directed RNA synthesis, inhibited the mobilization effect of PTH on bone. Kunin and Krane (1965) point out that enzymes such as collagenases may be required for the resorption of bone matrix and their production enhanced by PTH.

Over three decades ago, it was established that deficiency of vitamin D led to hypocalcemia (Thomson et al, 1932). It was believed that the D vitamins exerted their efforts by stimulating the parathyroid glands. Au et al (1965) demonstrated that vitamin D did not appear to be involved in any way in the control of PTH secretion by the serum calcium level. Deluca et al (1962) and Harrison et al (1964) established that vitamin D on its own could raise the serum calcium level but that PTH required the presence of vitamin D. Zull et al (1966), working on the relationship between vitamin D action and the actinomycin-sensitive process, suggested that vitamin D was required to alter the nuclear

membrane permeability to calcium which in turn increased synthesis of a calcium-carrier protein so that more calcium could be mobilized from bone. PTH would accelerate the synthesis of the calcium-carrier.

Effects of PTH on Other Organs. The action of PTH appears to be widespread throughout the body because effects on a variety of tissues other than bone have been reported. PTH control of the renal excretion of phosphate is a well established fact (Greenwald, 1911, Thomson et al, 1932 and Greep, 1955). Nicholson (1959) and Rasmussen and Deluca (1963) demonstrated that the hormone increased the rate of phosphate secretion by the distal tubule. Talmage (1956) and Kleeman et al (1958) found that there was an increased excretion of calcium in the urine of rats following parathyroidectomy and a decrease in excretion of calcium in the urine of rats when parathyroid extract was added to a hypoparathyroid animal. In more recent work, Wildrow et al (1962) demonstrated that PTH caused an increased calcium reabsorption in the renal tubules.

The effects of parathyroid extract on the incorporation of subsequently injected radioactive phosphate ($^{32}P_i$) into various phosphate fractions of the rat kidney were investigated by Egawa and Neuman (1964). The extract increased the incorporation of $^{32}P_i$ into total phosphate, acid soluble phosphate, inorganic phosphate, acid soluble

organic phosphate and nucleic acid phosphate of rat kidney.

Earlier investigators believed that PTH had little or no effect upon the intestinal absorption of calcium and phosphate (Rasmussen, 1961). Talmage and Elliott (1958), working with rats, found that parathyroidectomy two to four hours before the measurements were carried out led to a significant decrease (approximately 50%) in the rate of absorption of radiocalcium from an isolated loop of small intestine *in vivo*. Rasmussen (1959) and Cramer (1963) obtained similar results. Borle et al (1963) found that the addition of parathyroid extract increased the influx of phosphate in duodenal loops of rats *in vitro*.

A possible effect of PTH upon mammary glands was reported by Munson et al (1954). They obtained data indicating that the calcium content of milk decreased during the hyperparathyroidism induced by low calcium diets given to lactating rats. Parathyroidectomy abolished this response.

Clark (1939) demonstrated that the calcium content in lens decreased if it was incubated *in vitro* with parathyroid extract. In 1962, Firschein reported that parathyroidectomy reduced glucose uptake and lactate production by lenses during subsequent *in vitro* incubation and that addition of PTH restored the rate of glycolysis to the control level.

In its action on all of the above organs, it may be seen that the effects of PTH could result in an increase in the calcium and a reduction in the phosphate levels of serum.

Effects of PTH on Mitochondria. In more recent studies, effects of PTH have been studied at a subcellular level. Liver mitochondria have been most widely used.

Both vitamin D and PTH, added *in vitro*, but not related sterols or other peptide hormones, stimulated the release of calcium by isolated rat kidney mitochondria (Deluca et al, 1962). The PTH effect could be demonstrated only in the presence of vitamin D, whereas the action of the vitamin did not require the presence of hormone.

PTH stimulated the *in vitro* accumulation of inorganic magnesium phosphate by isolated rat liver mitochondria in the absence of vitamin D (Sallis et al, 1963). A respiratory stimulation accompanied the translocation of ions. In this system, phosphate could be replaced by sulfate or arsenate (Rasmussen, Sallis et al, 1964) and magnesium by manganese (Fang and Rasmussen, 1964). PTH could also enhance an *in vitro* potassium ion uptake into mitochondria that was respiratory supported (Rasmussen, Fischer et al, 1964). It was postulated by a number of investigators (some of these are Sallis et al, 1964, Fang and Rasmussen, 1964, Kimmich and Rasmussen, 1966) that PTH stimulated ion transport by utilizing the energy of oxidative phosphorylation prior to the entrance of inorganic phosphate into the phosphorylative chain. Uncoupling of oxidative phosphorylation by PTH without any net accumulation of phosphate or magnesium ions has been reported (Fang and Rasmussen, 1964 and Sallis et al,

1966). In the absence of magnesium and phosphate, PTH stimulated the in vitro mitochondrial respiration rate (Sallis et al, 1966). This response was completely blocked by the addition of magnesium but not phosphate and did not appear to be related to ion transport. However, when phosphate was added to the magnesium inhibited system, the respiratory response to hormone was restored and the respiration coupled to translocation of magnesium phosphate. Sallis et al suggested that magnesium was necessary to couple the PTH response to ion translocation in the mitochondria, and that, in its absence, the hormone acted as an uncoupler of oxidation from phosphorylation.

Kimmich and Rasmussen (1966) found that the terminal portion of the respiratory chain, isolated by an antimycin A block, was capable of supporting in vitro PTH-stimulated ion accumulation in mitochondria. Ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine were employed as electron donors to cytochrome C.

In addition to stimulating respiratory-supported ion transport, it has been established that PTH induces rapid and extensive mitochondrial swelling (Rasmussen, Fischer and Arnaud, 1964 and Utzumi et al, 1966). The PTH-induced swelling was accompanied by an increase in respiration and occurred in a medium containing only sucrose, respiratory substrate and Tris buffer (Utzumi et al, 1966). If magnesium and phosphate were added this swelling was reduced but if

phosphate or arsenate were added separately it was greatly increased. Utzumi et al suggested that the swelling process could be due to an increase in osmotic pressure resulting from ion accumulation in response to PTH. The minimal swelling when magnesium and phosphate were present could be due to a reduction in the internal osmotic pressure of the mitochondria resulting from the precipitation of magnesium phosphate.

Sallis and Deluca (1964) found that PTH, added in vitro, stimulated mitochondrial ATPase activity and depressed ATP-P_i exchange activity. However, no effect of PTH on phosphate accumulation by the mitochondria could be demonstrated using the incubation conditions necessary for the maximal hormonal stimulation of ATPase activity.

Aurbach, Houston et al (1964) reported that PTH, added in vitro to liver or kidney mitochondria, increased the formation of $^{14}CO_2$ from radioactive succinate by stimulating the oxidation of NADH produced by succinate. This effect was discernible at hormone concentrations of 7×10^{-9}M to 5×10^{-6}M.

Aurbach et al (1965) made the discovery that several non-hormonal basic proteins such as polylysine and protamine sulfate could duplicate all of the above effects of PTH on mitochondria. They concluded that further rigorous studies were necessary to determine whether any of the effects of PTH on the mitochondria could be responsible for the in vivo action

of the hormone. The finding of Aurbach et al, taken together with the fact that relatively large concentrations of hormone are required to produce them, leads one to question the physiological significance of the mitochondrial effects of PTH.

2) Metabolic Behavior of Isolated Rat Liver Cells

Several methods have been used by earlier workers to obtain cell suspensions from solid tissues. Jacob and Bhargava (1962) classified these methods as follows: (a) methods involving only a mechanical treatment of the tissue; (b) methods in which a mild mechanical treatment of the tissue is preceded by perfusion with a chelating agent to remove the calcium from the intracellular cement; (c) methods in which the mechanical treatment of the tissue is preceded by perfusion with a non-chelating agent; (d) methods in which the intracellular cement is softened or removed by a chemical or physical treatment not involving perfusion; and (e) methods based on an enzymatic treatment of the tissue to degrade polymeric components of the intracellular cement. All of these methods, except the ones in (b), require rather drastic physical treatment of the tissue and give low recovery of cells in the final suspension. The methods in (b) are based on an elegant technique developed by Anderson (1953). He perfused rat liver with calcium-free Locke's solution containing one of the following calcium binding agents,

citrate, pyrophosphate, versene or ATP. The perfused liver was homogenized gently in a Pyrex tube containing a loose-fitting lucite pestle. The crude cell suspension was strained through 10 XX bolting silk to remove clumps of cells and shreds of connective tissue, and then centrifuged several times to remove broken cells. Anderson's method was further improved by Jacob and Bhargava (1962) who used a loose-fitting pestle made from a soft rubber stopper. With this less drastic method of isolation, they obtained a higher recovery of unbroken cells.

It is probable that isolated liver cells are not in the native state because cell leakage has been shown to occur. Gibbons and Rienits (1961) found that cofactors such as ADP and NAD leaked out from dispersed cells. More recently, Ichihara, Adachi et al (1965) demonstrated that cofactors required for lipid synthesis, such as magnesium, manganese, ATP, GSH, NADPH, CoA, carbonate and citrate, also leaked out from isolated rat liver cells but not from tissue slices. Various enzymes, especially those of the soluble fraction of the cell, were also lost upon isolation of cells (Berry, 1962, Takeda et al, 1964 and Nutritional Reviews 24, 12, 1966). Enzymes that were almost completely lost included tryptophan pyrrolase, aldolase, lactic dehydrogenase, malic dehydrogenase, serine and threonine dehydrogenases, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase and glycolytic enzymes (the isolated

cells did not metabolize glucose, glycerol or fructose). In contrast, glucose-6-phosphatase, a microsomal enzyme, and the mitochondrial enzymes of the citric acid cycle were well retained.

Leakage from isolated cells could be due to an alteration of cell wall permeability caused by either a partial disruption of cell membranes, or removal of calcium, or both. From electron micrographs of liver cells isolated by similar methods, Berry et al (1962) concluded that partial disruption of cell membranes was a common occurrence while Leeson and Kalant (1961) could find no noticeable damage. Kalant and Miyata (1963) suggested that calcium or other divalent cations played some essential though unexplained role in the normal regulation of permeability by the cell wall. If, as seems likely, these ions were removed during perfusion of rat liver with a chelating agent, an alteration of cell wall permeability would occur.

3) Oxidative Phosphorylation

Although a great deal of work has been done, there is as yet no generally accepted scheme for coupling of respiration to phosphorylation of ADP. The types of approach utilized to attack this complex problem have included the use of a number of specific inhibitors, studies on "coupling factors" and attempts to isolate high energy intermediates. Some success has been reported in resolving particulate

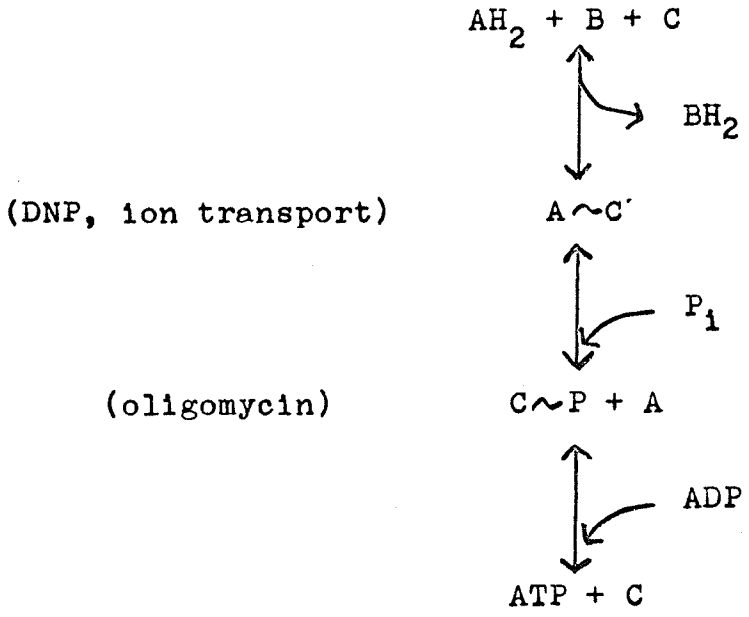
submitochondrial systems into two or more subfractions that are individually inactive in catalyzing oxidative phosphorylation but support respiration coupled to phosphorylation of ADP when they are combined. However, there are problems in interpreting the results. Restoration of phosphorylation could also be achieved by enzymatic removal of an endogenous inhibitor. It is a frustrating problem to damage mitochondrial preparations specifically and reproducibly in such a way as to obtain a useful assay system lacking only one coupling factor from one of the three known phosphorylation sites of the NAD-linked chain. Although some investigators (Peter and Boyer, 1963, Beyer, 1964 and Webster and Green, 1964) have isolated high energy compounds, phosphorylated proteins or lipids containing phosphohistidine, phosphoserine or other phosphoryl groups, there is not enough conclusive evidence to show that these function in oxidative phosphorylation (Kemp, 1966). In fact, Bieber and Boyer (1966) have recently presented evidence that these phosphorylated compounds do not serve as intermediates in the formation of ATP by oxidative phosphorylation in mitochondrial particles. The direct approach has therefore been so far unsuccessful but from results obtained in indirect ways using specific inhibitors, there is still no reason to doubt the existence of high energy intermediates.

In any attempt to devise a working model of oxidative phosphorylation and its interrelationship with

ion transport, the following facts must be considered. Investigators (Rossi and Lehninger, 1963, Lehninger et al, 1963 and Brierley et al, 1963) have found that oligomycin, a known inhibitor of the terminal reaction of the phosphorylative chain (Peter and Boyer, 1963 and Beyer, 1964), does not inhibit ion uptake in mitochondria when this is driven by a respiratory substrate but does inhibit when uptake is driven by ATP. They also demonstrated that respiratory inhibitors (amytal, antimycin and cyanide) and uncoupling agents (DNP and dicoumarol) inhibited ion uptake supported by respiration. However, only the uncouplers inhibited ion uptake when it was supported by ATP. Lardy et al (1958) showed that the ATPase action of DNP was blocked by oligomycin. Amytal, antimycin and cyanide are known to block electron transport between NAD and FAD, between cytochrome b and cytochrome c, and at cytochrome a respectively (Lehninger, 1964). Since there is no evidence that ATP supports ion transport or DNP-stimulated ATPase action except by a reversal of oxidative phosphorylation, the energy utilized for these purposes must be derived from the oxidative phosphorylation sequence prior to the point of oligomycin inhibition.

From the above facts, the working model that is favoured is shown below. In this model, AH_2 and B are two components of the electron transport chain and C is an additional component taking part in the reactions leading

to ATP. One of the intermediates is a high energy phosphate.



The site of action of DNP uncoupling and of energy utilization by ion transport would be at A~C and, therefore, in the presence of DNP, energy would not be available for ion transport. Oligomycin would inhibit at the terminal reaction by preventing the formation of C~P, or by binding with C~P, or by preventing the formation of ATP from C~P and ADP.

The above scheme does not agree with that presented by Lee and Ernster (1965). They suggested that DNP uncoupling occurred prior to ion transport but did not consider all the known facts. Thus, their scheme contained three high energy intermediates. They placed the site of action of octylguanidine between DNP and ion transport on the basis of the finding of Pressman (1963) that octylguanidine

inhibited the respiration of rat liver mitochondria with glutamate and malate as substrates and that DNP relieved this inhibition. However, they apparently did not give equal weight to the further finding of Pressman that octylguanidine did not inhibit DNP-induced ATPase action. If the DNP-induced ATPase can be considered a reversal of oxidative phosphorylation then octylguanidine and DNP would have to act at the same site and not at different sites as suggested by Lee and Ernster (1965). Acting at the same site, A~C in the present scheme, DNP would be a better uncoupler than octylguanidine is an inhibitor of oxidative phosphorylation. It is, of course, still possible that more than two high energy intermediates exist but what is definite is that at least two are required to explain the effects of DNP and oligomycin on ion transport.

MATERIALS

1) Chemicals

Acetone-dried parathyroid gland extract was obtained from Wilson Laboratories, Pharmaceutical Division, Wilson and Co. Inc., Chicago 9, Illinois.

Parathyroid extract (Lilly, U.S.P.) was obtained from Elli Lilly Co., Indianapolis, U.S.A.

Casein, alphacel, vitamin diet fortification mixture and calcium-free salt mixture were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

All nucleotides were obtained from P-L Biochemicals Inc., 1037 W. McKinley Ave., Milwaukee, Wisconsin.

Ouabain and ortho-dinitrophenol were obtained from Sigma Chemical Co., 3500 DeKalb St., St. Louis, Missouri.

Oligomycin was obtained from Mann Research Laboratories Inc., New York, New York. It consisted of 65 to 75% oligomycin A, 20 to 25% oligomycin B and 5 to 10% oligomycin C.

Antimycin A (B grade) was obtained from California Corporation for Biochemical Research, 3625 Medford St., Los Angeles, California.

Gramicidin N.F. and protamine sulfate were obtained from General Biochemicals, 900 Laboratory Park, Chagrin Falls, Ohio.

Poly L-lysine (molecular weights of 42,000 and 4,000) were obtained from Yeda Research and Development Co. Ltd.,

affiliated to the Weizmann Institute of Science, P.O. Box 26, Rheovoth, Israel.

Respiratory substrates L-malic acid (A grade), α-ketoglutaric acid, fumaric acid (C grade) and L-glutamic acid (A grade) were obtained from California Corporation for Biochemical Research, 3625 Medford St., Los Angeles, California.

Citric acid, succinic acid, sucrose and sodium cyanide were obtained from J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Sodium azide and D-mannitol were obtained from Fisher Scientific Co., Fairlawn, New Jersey.

Most other reagents were obtained either from Fisher Scientific Co., Fairlawn, New Jersey or from J.T. Baker Chemical Co., Phillipsburg, New Jersey.

2) Animals

The rats were obtained from a local colony of Long-Evans strain.

METHODS

1) Purification of PTH

The hormone was purified by a modification of the method of Aurbach and Potts (1964). The following is the procedure for purifying 50gr of crude powder.

Wilson acetone extract (50gr) was stirred into 600ml of 90% phenol (12ml per gr of extract) for two hours at room temperature. Five volumes (3,000ml) of a solution of glacial acetic acid-acetone (1:4), 0.004M in NaCl, was added and the mixture stirred for one hour in the cold (5°C). The large flocculent precipitate was removed by centrifugation at 5,900 x g for thirty minutes. The insoluble material was re-extracted with 300ml of the acetic acid-acetone-NaCl mixture and recentrifuged. The total supernatant (3,150ml) was mixed with an equal volume of ether and, after standing overnight in the cold, the precipitate was collected by centrifugation at 1,460 x g for fifteen minutes and washed three times with 50ml acetone. The wet weight of the precipitate was 35gr which reduced to 3.7gr when it was dried in a vacuum. The dried crude extract was dissolved in 74ml of cold 80% acetic acid (20ml per gr) and the mixture stirred for five hours. The suspension was centrifuged at 1,460 x g for fifteen minutes. The precipitate and floating lumps were homogenized with some of the supernatant and stirred again for fifteen minutes. Three volumes (250ml) of

cold deionized water was added and the mixture stirred for ten minutes. The medium was centrifuged for fifteen minutes at 10,400 x g to yield supernatant I (310ml) and precipitate I.

Cold 80% acetic acid (60ml) was added to precipitate I and the mixture stirred in the cold until all of the precipitate was dissolved. Cold deionized water (180ml) was added and the medium stirred for ten minutes and centrifuged at 10,400 x g for fifteen minutes to yield supernatant II (230ml) and precipitate II.

To supernatant I (310ml), 18.6gr NaCl (6%) was added and the medium stirred for thirty minutes. The suspension was centrifuged at 10,400 x g for fifteen minutes to yield supernatant Ia (294ml) and precipitate Ia.

To supernatant II (230ml), 13.8gr NaCl (6%) was added and the mixture stirred for ten minutes and centrifuged at 10,400 x g for fifteen minutes to yield supernatant IIa (224ml) and precipitate IIa.

Precipitates Ia and IIa were bulked with 60ml of cold 80% acetic acid and the mixture stirred for three hours. Cold deionized water (180ml) and 14.4gr NaCl (6%) were added. The mixture was stirred for thirty minutes and centrifuged at 10,400 x g for twenty minutes to yield supernatant IIIa (238ml) and precipitate IIIa. To supernatant IIIa, 7.14gr TCA (3%) was added and the mixture stirred for fifteen minutes. After standing for forty-five minutes, it was centrifuged at 10,400 x g for fifteen minutes to yield supernatant IIIb

and precipitate IIIb.

To supernatant Ia (294ml), 8.82gr TCA (3%) was added and the mixture stirred for fifteen minutes. To supernatant IIa (224ml), 6.72gr TCA (3%) was added and the mixture stirred for fifteen minutes. The two solutions were left to stand overnight and then centrifuged at 10,400 x g for fifteen minutes to yield supernatant Ib, precipitate Ib, supernatant IIb and precipitate IIb respectively.

Twenty ml of 3% TCA was added to each of precipitates Ib, IIb and IIIb which were then pooled and centrifuged at 10,400 x g for twenty minutes to yield supernatant IV and precipitate IV.

Precipitate IV was suspended in 37ml (10ml per gr of crude extract) of 0.02N HCl and extracted five times with 57ml (1.5 volumes) of peroxide-free ether each time. The ether was partially evaporated in a desiccator under vacuum. Four ml of AG-1-X4 (200 to 400 mesh) in the acetone form was added to the aqueous solution of hormone and it was shaken for five minutes. The solution was passed through an HA millipore filter to yield 36ml of a clear, pale-yellow solution which was made up to 40ml with deionized water and lyophilized.

The partially purified material, 230.29mg, was dissolved in 4.5ml of 0.2M ammonium acetate pH 4.7 and added to a 3 x 100cm Sephadex G-100 column that had been equilibrated with 0.2M ammonium acetate buffer pH 4.7.

The rate of elution of the column was 10 to 12ml per hour. One hundred and forty-eight fractions of 4ml each were collected and the optical density at 277mμ was measured using a Beckman DU spectrophotometer. Fractions 75 to 94 were pooled and lyophilized to yield the purified hormone. The hormone was separated into ten bottles each containing 2.7mg (approximately 8,000 units) of purified PTH and stored in a desiccator at -20°C. The purified hormone was assayed against standard parathyroid extract (Lilly, U.S.P.) by measuring the calcium in the serum of parathyroidectomized rats after injection. Seventeen μg of the purified hormone was found to contain approximately fifty units (one unit of parathyroid hormone is defined as 1/100 of the amount required to cause an increase of 5mg of calcium in the blood serum of a twenty kilogram dog, the increase being determined fifteen hours after injection).

The purity of this hormone preparation was checked by polyacrylamide gel electrophoresis. One major and two minor bands were seen indicating inhomogeniety of the preparation. At present, these bands have not been characterized.

2) Preparation of Calcium-free Diet

Calcium-free diet contained the following ingredients; 1,000gr starch, 480gr casein, 60gr alphacel, 40gr vitamin mixture, 30gr calcium-free salt mixture, 100gr corn oil, 260gr sucrose and 300ml distilled water.

The following ingredients were mixed separately; a) starch and casein, b) alphacel, vitamin mixture and salt mixture and c) corn oil and sucrose. The combined mixture of a) and b) were stirred in slowly with the corn oil and sucrose. After mixing as well as possible, the distilled water was added and mixing continued. The final mixture was sifted two times through a hand sieve in order to form a fine mixed powder.

3) Parathyroidectomy Procedure

Long-Evans rats of known weight and sex were fed the calcium-free diet for four to five days prior to parathyroidectomy. The animals were anaesthetized with ether. The parathyroid glands were exposed and destroyed by electrocautery. The incision was closed with Michel clips and the animal allowed to regain conciousness. One and one-half hours after parathyroidectomy, PTH in 30% gelatin was injected subdermally. Blood samples from the tail were collected in heparinised capillary tubes prior to and after parathyroidectomy. The tubes were centrifuged immediately and the plasma analyzed for calcium.

4) Direct Microdetermination of Serum Calcium

Principle of the Method. Glyoxal-bis(2-hydroxyanil), a Schiff base, forms coloured complexes with metals. The red calcium complex may be extracted with chloroform. Other

metal complexes are either not dissolved by chloroform or may be removed by addition of alkaline carbonate, cyanide or alkaline buffer. Goldstein and Stark-Mayer (1958) tested the reagent with a wide range of metal ions. The ease of removing metal complexes other than calcium makes glyoxal-bis specific for calcium.

Materials.

Stock calcium standard solution (100µg Ca/ml). 0.2498gr $CaCO_3$ was dissolved in 7ml of 1N HCl and diluted to one litre with deionized water.

Working calcium standard solutions of 12.5, 6.25, 5.0, 2.5 and 1.25µg Ca/ml were made by dilution of the stock solution.

Glyoxal-bis(2-hydroxyanil) (0.5%). 0.5gr of glyoxal-bis was dissolved in 100ml of absolute ethyl alcohol with vigorous shaking.

Stock carbonate-free 2.0N NaOH. 50% NaOH was made using deionized water. The sodium carbonate precipitate that formed was removed by centrifugation. The supernatant was diluted to 2N and standardized against potassium hydrogen phthalate using phenolphthalein as indicator. The solution was then corrected to 2.0N.

Working NaOH solutions of 0.5N and 1.2N were made by dilution of the stock carbonate-free 2.0N NaOH.

Experimental Procedure. Blanks (200µl deionized water), standards (200µl working calcium standards) and serum samples (20µl serum added to 200µl of deionized water) were placed in duplicate into 10 x 75mm Coleman tubes which were set into a distilled water ice bath at 0°C. Glyoxal-bis (200µl) was added to all tubes and the ingredients mixed on a vortex mixer. The tubes were cooled for several minutes in the ice bath and then reagitated. Fifty µl of 0.5N NaOH was added to the blanks and standards while 50µl of 1.2N NaOH was added to the serum samples. The tubes were mixed individually and returned to the ice bath where a pale yellow or very pale pink colour was allowed to develop for fifteen minutes. Ice cold chloroform (500µl) was added to each tube. All tubes were closed with clean, calcium-free, white rubber stoppers and shaken vigorously twenty to thirty times. The tubes were centrifuged in a cold clinical centrifuge for twenty seconds at full speed. The tubes were then placed in a water bath at 10 to 15°C, wiped dry and read at 540mµ in a Klett-Summerson photometer (industrial model) fitted with a microadaptor. The values obtained in Klett units for the working calcium standard solutions from four different experiments were averaged and a standard deviation was determined for each calcium concentration. These were 64 ± 4, 119 ± 5, 243 ± 2, 289 ± 8, 570 ± 9 Klett units for 0.25, 0.50, 1.00, 1.25, 2.50µg Ca/sample respectively. The standard curve of µg Ca/sample versus Klett units may

be seen in figure 1.

5) Determination of Inorganic Phosphate

Principle of the Method. The method utilized was that described by Chen, Toribara and Warner (1956) which depends upon the formation of a phosphomolybdate complex and the reduction of this with ascorbic acid to yield a stable coloured complex. It is very sensitive and as little as 0.15µg of phosphorus can be detected.

Materials.

Ammonium molybdate (2.5%). 2.5gr was dissolved in 100ml of deionized water.

Sulfuric acid (6N). 95.5% pure H_2SO_4 was diluted to 6.0N.

Ascorbic acid (powder).

Phosphate reagent. 5ml of 2.5% ammonium molybdate, 5ml of 6N H_2SO_4 and 0.5gr ascorbic acid were mixed and made up to 25ml with deionized water.

Stock phosphate solution (100µg/ml). 0.4383gr KH_2PO_4 was dissolved in one litre of 0.1N H_2SO_4.

Working phosphate solutions of 0.25, 0.5, 1.0 and 2.0µg phosphorus/ml were made by dilution of the stock solution.

Experimental Procedure. Blanks and standards consisted of 500µl deionized water and 500µl standard solutions

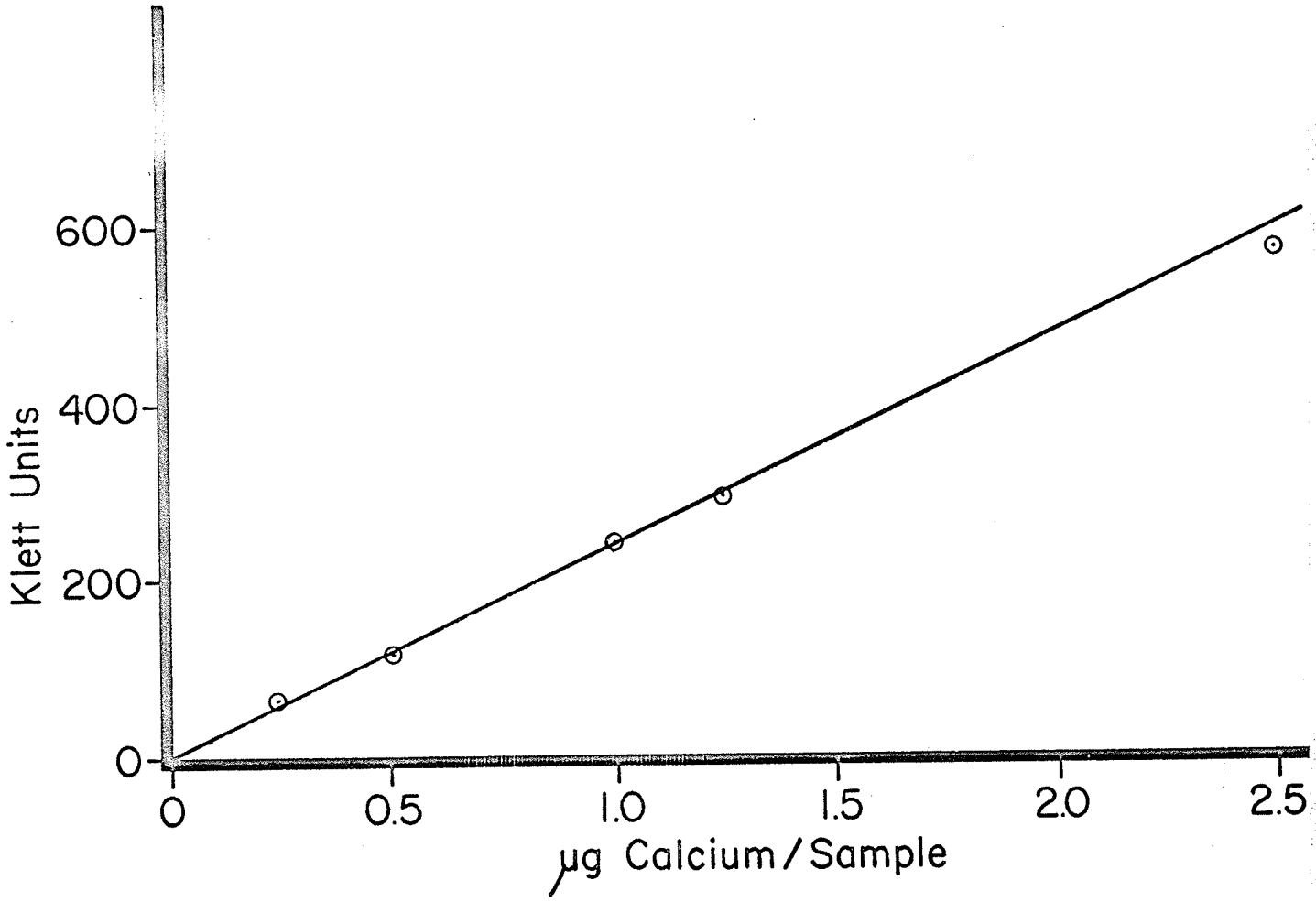


Figure 1. Calcium method-standard curve.

respectively. Suitable aliquots of solutions to be analyzed for phosphate were taken and made up to 500µl with deionized water. 10 x 75mm test tubes were used in all cases. Phosphate reagent (500µl) was added to all tubes. After mixing, the tubes were placed in a 37°C water bath for one hour. The tubes were removed, cooled to room temperature and the absorbance of the solutions read on a Beckman DU spectrophotometer at 820mµ. The values from twenty-four different experiments were averaged and a standard deviation was determined for each phosphate concentration. These were 0.097 ± 0.002, 0.199 ± 0.001, 0.402 ± 0.002, 0.793 ± 0.005 O.D. units for 0.125, 0.250, 0.500, 1.000µg phosphorus/sample respectively. The standard curve of µg phosphorus/sample versus optical density may be seen in figure 2.

6) Determination of Inorganic Nitrogen

Principle of the Method. The method utilized was a modified version of that outlined by Hawk, Osler and Summerson (1950) in which various forms of nitrogenous substances are converted to ammonium ion by oxidation with boiling concentrated sulfuric acid. The ammonium ion is reacted with Nessler's reagent to form a stable coloured complex.

Materials.

Concentrated H_2SO_4.

Hydrogen peroxide (30%).

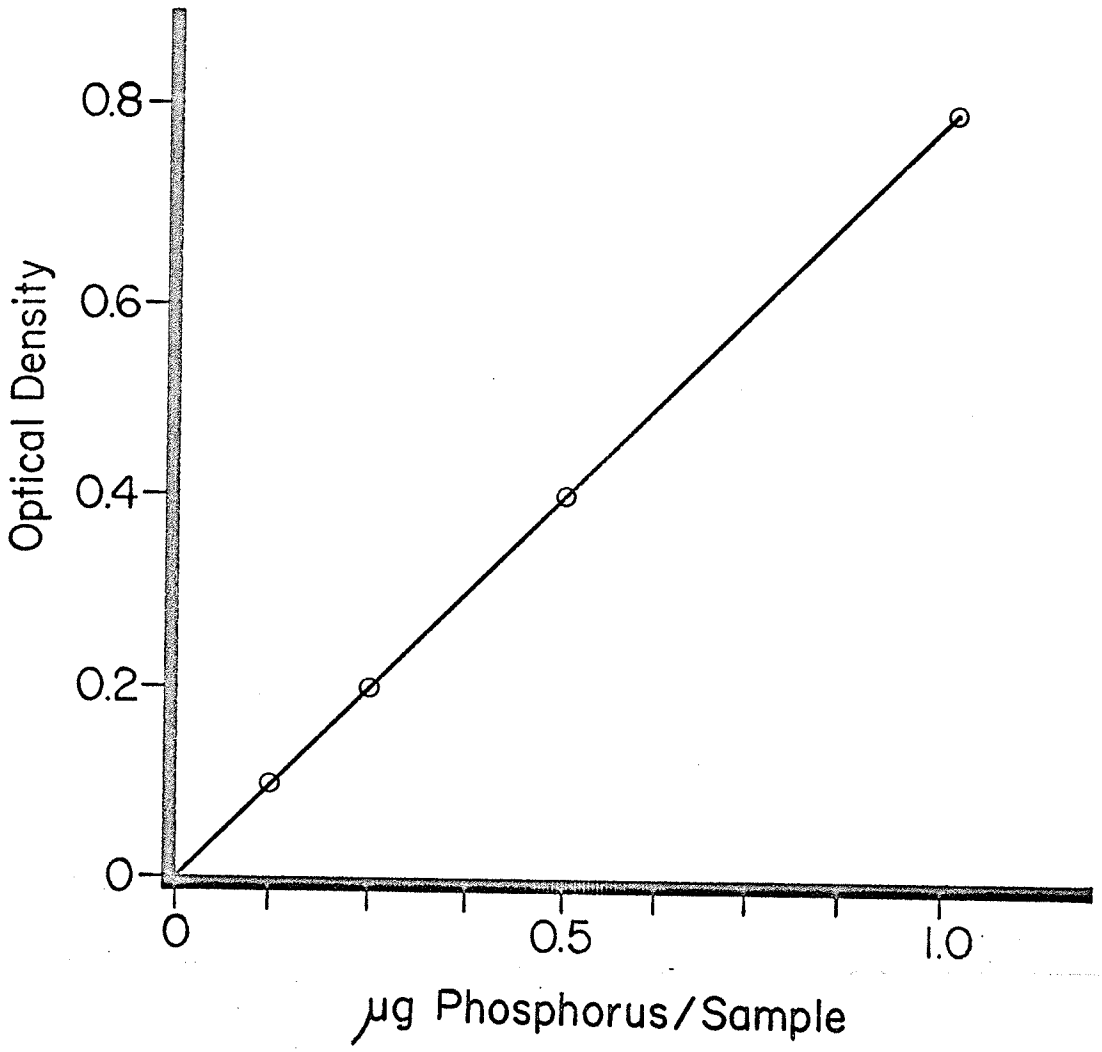


Figure 2. Phosphate method-standard curve.

Stock Nessler's reagent. 150gr KI and 110gr iodine were placed in a 500ml Erlenmeyer flask with 100ml deionized water. To this, 150gr of metallic mercury was added and the medium shaken in the cold until the red colour of the iodine was replaced by the green colour of the double iodide. The solution was decanted from the mercury, the mercury washed and the washings combined with the solution and diluted to two litres with deionized water.

Working Nessler's reagent. 175ml of stock Nessler's reagent was added to 600ml of water containing 70gr of dissolved NaOH. The mixture was made up to one litre with deionized water.

Stock nitrogen standard (100µg/ml). 0.47gr $(NH_4)_2SO_4$ and 125ml concentrated H_2SO_4 were made up to one litre with deionized water.

Working nitrogen standards of 50, 40, 25, 10 and 5µg nitrogen/ml were made by dilution of the stock solution using 4.5N H_2SO_4.

Experimental Procedure. Suitable aliquots of the sample to be analyzed were digested with 1.0ml of concentrated H_2SO_4 in 50ml Kjeldhal flasks for at least one hour. Eight drops of 30% hydrogen peroxide were added and the heating continued for fifteen minutes. After cooling, 2.0ml of 4.5N H_2SO_4 were added and the sample transferred to a 10ml volumetric flask and made up to the mark with deionized

water. The nitrogen was now present as $(NH_4)_2SO_4$ in 4.5N H_2SO_4. Aliquots of 250µl were removed to 10 x 75mm test tubes in ice and 1.0ml of cold working Nessler's reagent added. After mixing, the tubes were left for five to ten minutes in the cold and then for one-half hour at room temperature. The absorbance of the solutions was read on a Beckman DU spectrophotometer at 465mµ. The values from fourteen different experiments were averaged and a standard deviation was determined for each nitrogen concentration. These were 0.097 ± 0.003, 0.202 ± 0.002, 0.550 ± 0.031, 0.908 ± 0.010, 1.156 ± 0.012 O.D. units for 1.25, 2.50, 6.25, 10.00, 12.50µg nitrogen/sample respectively. The standard curve of µg nitrogen/sample versus optical density may be seen in figure 3.

7) Isolation of Mitochondria from Rat Liver

The method utilized was that reported by Deluca and Engstrom (1961). Fresh livers were obtained from rats of known weight and sex that had been stunned by a blow on the head and quickly exsanguinated. The livers were plunged into ice cold 0.34M sucrose solution. The connective tissue was removed and the livers were weighed in tared beakers containing ice cold 0.34M sucrose. A 10% homogenate (5gr of liver and 45ml of 0.34M sucrose) was prepared by mincing with scissors and then homogenizing in a Potter-Elvehjem homogenizer fitted with a teflon pestle. Twenty ml of

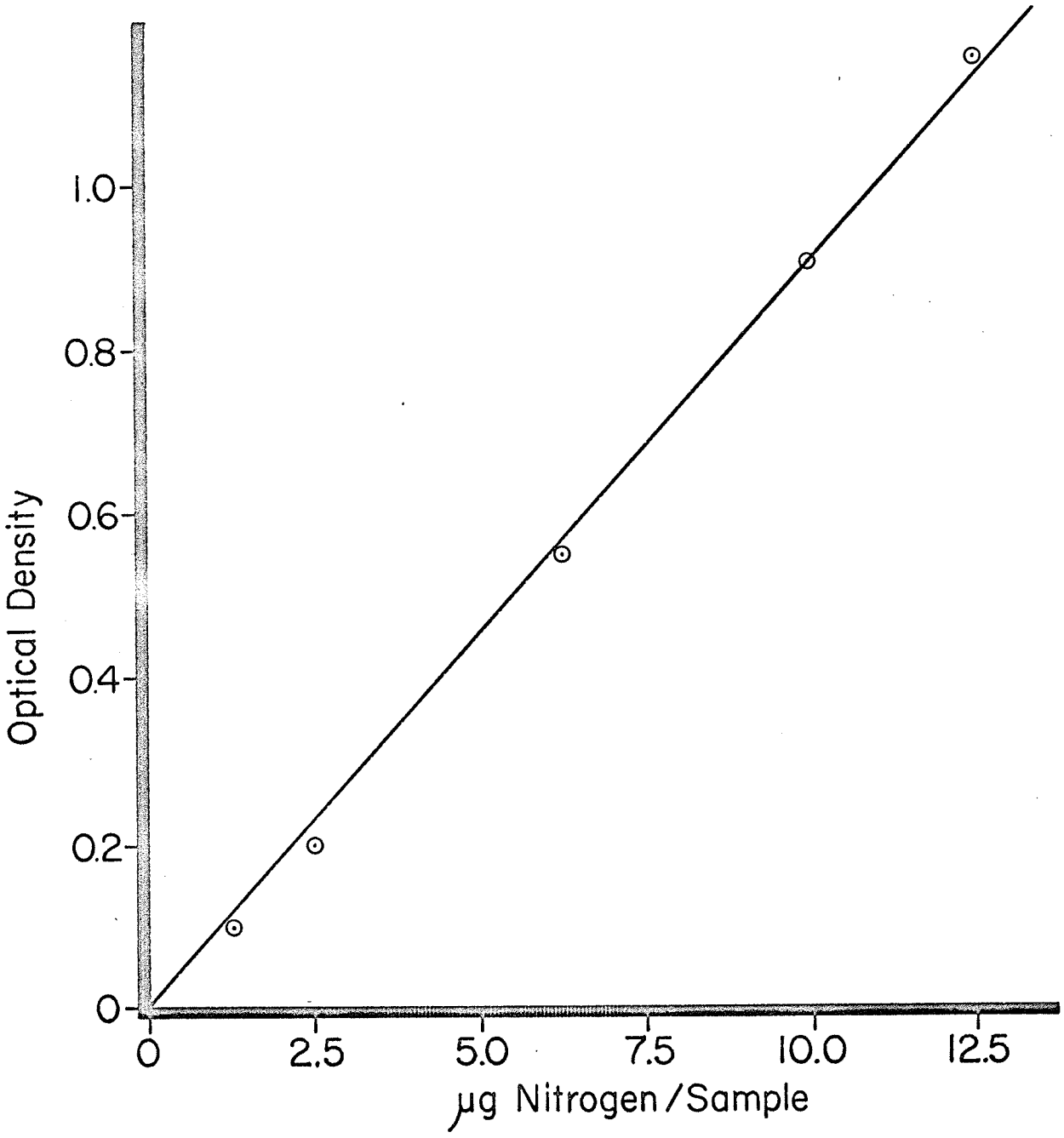


Figure 3. Nitrogen method-standard curve.

homogenate was layered over 20ml of 0.34M sucrose and centrifuged at 600 x g for ten minutes to remove nuclei, debris and unbroken cells. The upper layer was carefully transferred into another centrifuge tube and recentrifuged at 8,000 x g for ten minutes to sediment the mitochondria. The supernatant was decanted, the sides of the tube wiped dry and the mitochondria washed once with 20ml of 0.25M sucrose. The mitochondria were resedimented by centrifugation at 8,000 x g for ten minutes. The supernatant was decanted and the mitochondria suspended in 10ml of 0.34M sucrose. The total time required for isolation was approximately two hours. Aliquots (0.5ml) of the mitochondrial suspension were analysed for nitrogen. The average value obtained from fifty isolations was 0.403 ± 0.010mg (s.d.).

8) Determination of Phosphate Uptake in Mitochondria

The method utilized was that described by Sallis, Deluca and Rasmussen (1963). The incubation medium consisted of 13.3mM sodium phosphate pH 7.2, 300mM sucrose, 10mM sodium glutamate pH 7.2, 6.7mM $MgCl_2$, 1.0ml of mitochondrial suspension (0.8mg of nitrogen) and deionized water in a total of 3.0ml. The incubation was performed in 10ml Erlenmeyer flasks in a shaking water bath at 30°C. PTH (250units), dissolved in 200µl 0.002N acetic acid, was added after a five minute pre-incubation period to the experimental flasks, 200µl of 0.002N acetic acid to the

controls, and the incubation continued for a further twenty-five minutes. The mitochondria were removed by filtration on a 30mg celite pad (see figure 4) layered over Whatman #1 paper contained in a one inch diameter Millipore filter holder. The pad was washed two times with 1ml of 0.34M sucrose each time. The pads were transferred to a small beaker and dried at 110°C for one hour. Three ml of 5N H_2SO_4 was added to extract the inorganic phosphate from the pad. After standing for one half-hour, the sample was centrifuged and the supernatant was analyzed for inorganic phosphate.

9) Preparation of Rat Liver Cell Suspensions

The method utilized was a modification of the method used by Jacob and Bhargava (1962). A rat of known weight and sex was stunned by a blow on the head and quickly exsanguinated. The liver was perfused in situ through the inferior vena cava with approximately 50ml of cold calcium-free Locke's solution (0.154M NaCl, 0.0056M KCl, 0.001M $NaHCO_3$, 0.0055M glucose) containing 0.027M sodium citrate pH 7.2. The pressure was gradually increased during perfusion until the blanched liver was fully distended. As soon as the perfusion was complete, the liver was excised, washed, blotted and weighed. All subsequent operations were carried out in the cold. A homogenate of 5gr of liver and 25ml of cold calcium-free Locke's solution containing citrate was prepared by mincing

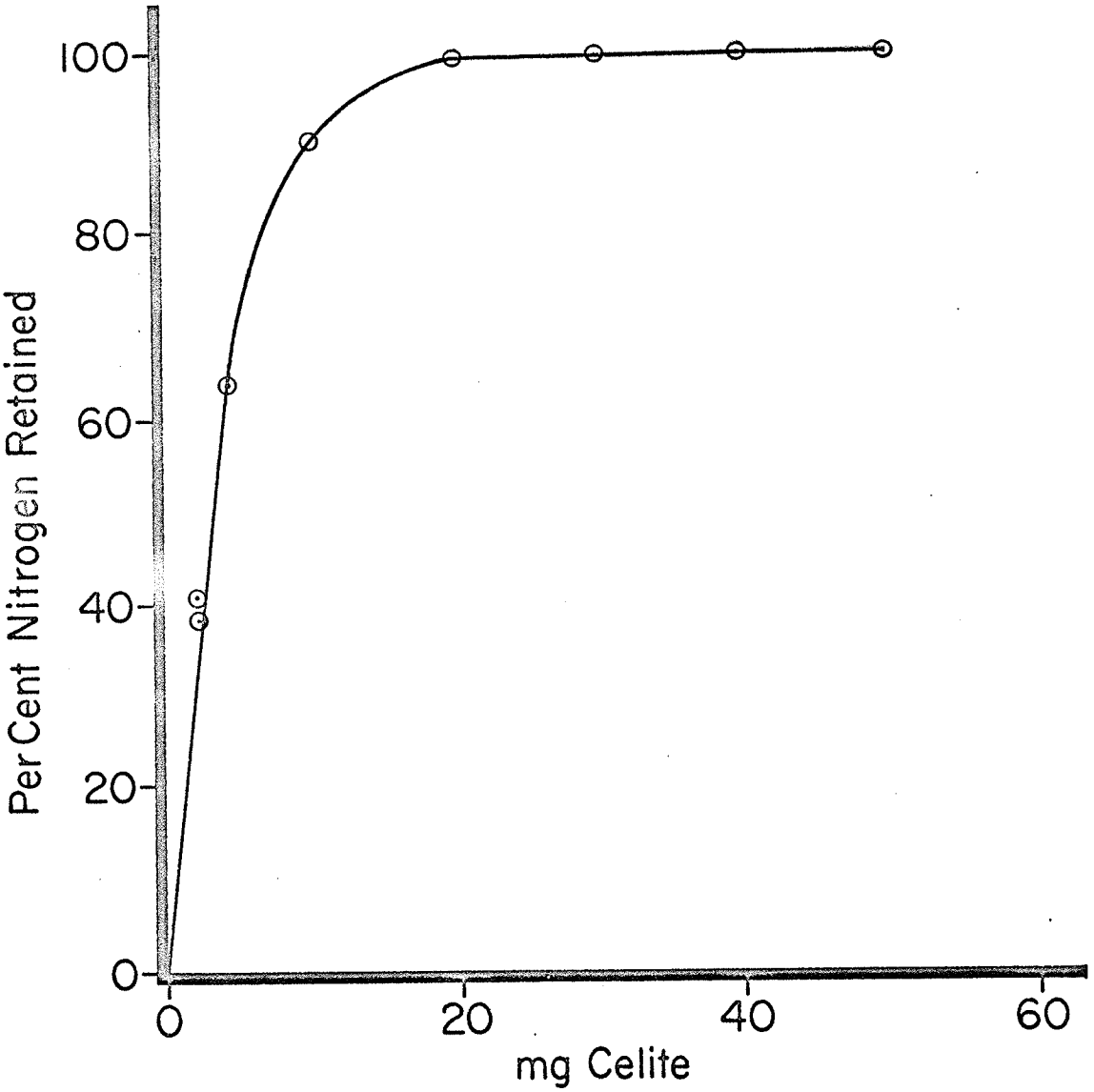


Figure 4. Recovery of mitochondria on celite filter.

with scissors and then homogenizing in a modified Potter-Elvehjem homogenizer with a loose-fitting pestle made from a soft rubber stopper. Twenty-five up and down strokes of the pestle by hand were found to be sufficient to disperse the cells. The homogenate was filtered once through two layers of nylon net (approximately thirty-five mesh) to remove strands of connective tissue and clumps of cells. Twenty ml of the filtrate was centrifuged at 100 to 200 x g for two minutes to remove cell debris and blood cells. The supernatant was decanted and the cell sediment suspended in 20ml of calcium-free Locke's solution containing citrate and recentrifuged. The supernatant was again decanted and the cell sediment resuspended in 10ml of calcium-free Locke's solution containing citrate. Cell yield was estimated in three ways:

a) Cell Counting. A range of 2 to 4 x 10^6 cells/ml was obtained by direct count on undiluted suspension in a hemocytometer. Campbell and Kosterlitz (1952) found that the DNA content of rat liver was 2.13gr/1,000gr moist fresh tissue and that 11mg of DNA was equivalent to 10^9 cells for rats in a weight range of 100 to 340gr. It can be calculated from this data that 1gr of liver contains 200 x 10^6 cells. On this basis and allowing for dilution during isolation, the maximum yield of 4 x 10^6 cells/ml of suspension is equivalent to a recovery of only 6% of the cells in the original liver tissue. The most likely reason for this low

recovery was incomplete dispersion. Large clumps of cells, which were removed by the nylon filter, were observed in the homogenate.

b) Milligrams of Cellular Nitrogen. Estimation by Nessler's method yielded values for nitrogen content of 0.28 to 0.56mg/ml of suspension.

c) Milligrams Dry Weight. 1.0ml of cell suspension was dried at 110°C for one hour. Values ranged between 4 and 8mg dry weight/ml of suspension.

10) Determination of Respiration Rate of Isolated Cells

Oxygen uptake of isolated cells was determined using the techniques and conventional Warburg apparatus as described by Umbreit, Burris and Stauffer (1959). The manometers contained Brodie's fluid and the conical flasks used were of 14ml capacity and equipped with two sidearms.

The basic incubation medium devised by the author consisted of 300mM mannitol, 20mM sodium phosphate buffer pH 7.2, 15mM sodium succinate pH 7.2 and 1mM $MgCl_2$ in deionized water and contained isolated cells (1 to 2 x 10^6 in 0.5ml of calcium-free Locke's solution containing citrate) in a total volume of 2.0ml. The final concentrations of the components added with the cell suspension were 39mM NaCl, 1.4mM KCl, 0.25mM $NaHCO_3$, 1.4mM glucose and 6.7mM sodium citrate. The medium was added to the main compartment of the Warburg flask and 200µl of 20% KOH and a fluted filter paper

strip were added to the center well to absorb CO_2. After addition of cells, the flasks were incubated at 30°C with air as the gas phase and were shaken at 100 cycles/minute to promote rapid gas exchange. After a five minute preincubation period, manometer readings were taken at ten minute intervals using the 15cm mark on the closed side of the manometer as a reference point.

Oxygen consumption was calculated using the formula, $x = hk_{O_2}$, where x is the amount of gas utilized in µl, h the alteration of reading in mm on the open arm of the manometer and k_{O_2} the flask constant. Flask constants for oxygen were calculated using the formula, $k_{O_2} = (V_g + 273/T)/P_o + (V_f \alpha)/P_o$, where α is the solubility coefficient of oxygen (0.026 in water), V_g the total gas volume of flask and connecting tubes, V_f the volume of fluid in the flask, P_o the barometric pressure (10,000mm for Brodie's fluid) and T the absolute temperature. Changes in P_o due to variations in barometric pressure and bath temperature were corrected for by using a thermobar which consisted of a flask containing 2.0ml of water.

The cellular respiration rate in the basic incubation medium was found to be 60 $\mu l O_2/hr/10^6$ cells (figure 5) and was called the <u>control respiration rate</u>.

11) <u>Oxidation and Reduction of PTH</u>

The method used was that described by Tashjian,

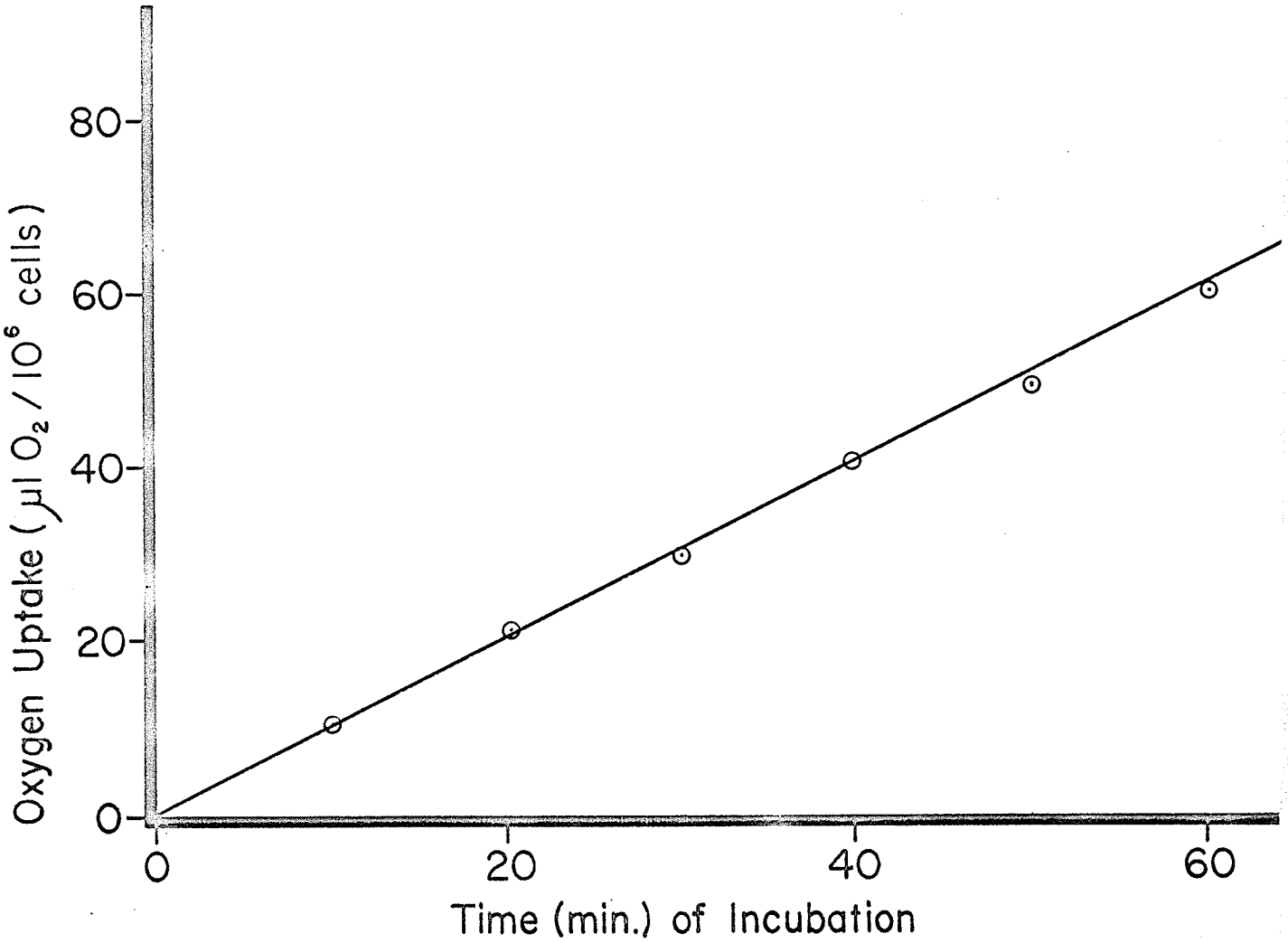


Figure 5. Determination of control respiration rate for isolated cells.

Ontjes and Munson (1964). PTH (400 units) was incubated with 100µl of 0.1M hydrogen peroxide at pH 3.8 for thirty minutes at 25°C. Cold deionized water (100µl) was then added. A portion of the oxidized hormone was reduced again by incubating 100µl of the above solution with 100µl of 0.12M cysteine hydrochloride for three hours at 80°C. The samples were then lyophilized and dissolved in 100µl of deionized water.

12) Estimation by Paper-Strip Electrophoresis of Interconversion of Adenosine Nucleotides by Isolated Cells

Isolated cells (1 to 2 x 10^6 in 0.5ml of calcium-free Locke's solution containing citrate) were incubated at 30°C for thirty minutes in a medium containing 300mM mannitol, 20mM sodium phosphate pH 7.2, 15mM sodium succinate pH 7.2, 5.0mM adenosine nucleotide, 1mM $MgCl_2$ and deionized water in a final volume of 2.0ml. The reaction was terminated by centrifuging in the cold and the supernatant immediately submitted to paper-strip electrophoresis by the method described by Sato, Thomson and Danforth (1963). Ten µl was applied with a sample applicator to 3 x 30.5cm Beckman-Spinco filter paper strips previously moistened with electrophoresis solution (0.05M citrate buffer pH 3.7 was made by dissolving 14.7102gr of citric acid and 8.6957gr of sodium citrate in two litres of deionized water). Electrophoresis was carried out in a Beckman-Spinco Model R at 400 volts for three and one-half hours. The strips were dried and observed under

ultraviolet light to locate the nucleotides ATP, ADP and AMP. These were eluted by boiling for thirty minutes with 4.0ml of 1.0N HCl. The samples were read directly on a Beckman DU spectrophotometer at 257mμ. The amount of nucleotide present was obtained by the use of the molar indexes of 15.1, 14.9 and 14.7 x 10^3 for AMP, ADP and ATP respectively (Bock et al, 1956).

RESULTS

Mitochondrial Phosphate Uptake

It is well known that, under certain conditions, *in vitro* mitochondrial accumulation of phosphate can occur (Lehninger et al, 1963 and Brierley et al, 1963). Representative data obtained in the present study with rats of widely differing weights is given in table 1. No data on the effect of rat weight on mitochondrial phosphate uptake could be found in the literature. It may be seen from the results that nitrogen content of the mitochondrial preparations and phosphate uptake per mg of mitochondrial nitrogen were not dependent on rat weight.

Sallis et al (1963) demonstrated that PTH at a concentration of 28µg/ml stimulated the mitochondrial accumulation of phosphate by approximately 1µMole phosphate/mg nitrogen. This finding was confirmed in the present work (table 2), however in contrast to the study of Sallis et al, PTH at concentrations greater than 28µg/ml did not further stimulate the phosphate accumulation.

Isolation of Cells

Anderson (1953) demonstrated that the presence of citrate in the medium used for perfusion increased the cell yield from rat liver. This finding was confirmed (table 3). Intracellular cement is generally considered to consist of a

TABLE 1.

Effect of Rat Weight on Mitochondrial Phosphate Uptake

Rat Weight (gr)	Mitochondrial Nitrogen (mg/ml)	Phosphate Uptake (μMoles/mg nitrogen)
59	0.796	0.48
164	0.788	0.49
350	0.820	0.46

TABLE 2.

Effect of PTH Concentration on Mitochondrial Phosphate Uptake

Concentration of PTH (μg/ml)	Phosphate Uptake (μMoles/mg nitrogen)
0	0.5
28	1.5
40	1.3
80	1.3

TABLE 3.

Effect of Presence of Citrate in Perfusion Medium on Yield of Isolated Cells

Concentration of Citrate	Cell Count ($\times 10^{-6}$) per ml of Final Suspension
0.000M	2.2
0.027M	2.8

slightly soluble calcium salt. Removal of this by chelation with citrate would result in a greater dispersion of the cells.

Anderson (1953) also reported that twenty strokes with a hand-driven pestle were sufficient to disperse rat liver cells while Jacob and Bhargava (1962) used ten to twelve up and down strokes. With the pestle constructed for the present study, varying the number of strokes from fifteen to fifty-five did not alter the cell yield (table 4).

Because no data could be found in the literature, the stability of isolated rat liver cells in various media was studied. They were found to be unstable (lysed) in sucrose or mannitol unless these solutions were decidedly hypertonic or the pH was below 6.3 but were stable in calcium-free Locke's solution containing citrate. This solution, which was the perfusion medium, was therefore used throughout the preparation of the cells.

On the basis of cell count, the dry weight and nitrogen content of the final cell suspensions were constant and did not depend on sex or weight of the animal (table 5). Assuming the water content of liver to be approximately 70%, the recovery of dry weight in the final suspensions varied from 4 to 8%. Assuming the cell content of liver to be 200×10^6/gr wet liver, the recovery of cells in the final suspensions varied from 3 to 6% (see Methods). The close agreement between these two calculations indicates that the

TABLE 4.

Effect of Duration of Homogenization on Yield of Isolated Cells

Number of Pestle Strokes	Cell Count ($\times 10^{-6}$) per ml of Final Suspension
15	1.8
25	1.6
35	1.6
55	1.8

TABLE 5.

Effect of Sex and Weight on Dry Weight, Nitrogen Content and Control Respiration Rate of Isolated Cells

Sex	Rat Weight (gr)	Dry Weight ($mg/10^6$ cells)	Nitrogen ($mg/10^6$ cells)	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)
M	115	1.9	0.12	58
F	121	2.1	0.14	60
M	250	2.0	0.12	60
F	225	2.3	0.11	60
M	340	2.0	0.14	66
F	365	2.0	0.14	58

cell suspensions were relatively free of debris. Gross absence of debris was confirmed by microscopic examination at 400 times magnification. Jacob and Bhargava (1962) claimed a dry weight recovery of 70 to 80%. However, it may be calculated from their data that the yield of cells was only 4 to 25%. The wider discrepancy between these two sets of figures indicates that their cell suspensions contained considerably more debris.

Various Effects on Respiration Rate of Isolated Cells

In the preparation of the cells a single washing step was included. This reduced the respiration rate somewhat but repeated washings had no further effect (table 6). Working with isolated mouse liver cells, Berry (1962) also found that one washing slightly diminished the respiration rate.

The respiration rate of isolated cells was independent of sex and did not vary over a wide range of body weight (table 5). Both male and female rats in a weight range of 180 to 280gr were used for preparation of cells.

Because of the variation in cell yield, the final concentration of cells in the incubation flask varied from 0.5 to 1.0 x 10^6 cells/ml. However, the respiration rate was found to be constant over a much wider range (table 7).

TABLE 6.

Effect of Cell Washing on Control Respiration Rate

Number of Washings	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)
0	70
1	61
2	61
3	61

TABLE 7.

Effect of Cell Concentration on Control Respiration Rate

Cell Concentration (cells/ml x 10^{-6})	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)
0.18	58
0.26	60
0.44	62
0.68	62
0.88	59
1.26	57
1.75	59
2.22	57

Optimal Requirements for Respiration Rate of Isolated Cells

The incubation medium used was modified from one devised by Ichihara, Tanioka and Takeda (1965) for the measurement of respiration rates of isolated rat liver cells. Their medium contained 350mM mannitol, 50mM glucose, 20mM sodium phosphate pH 7.2, 10mM KCl, 2.5mM sodium succinate pH 7.2, 1mM $MgCl_2$ and 0.15mM ADP.

To avoid clumping, a cell concentration below 2×10^6 cells/ml was used. With cell concentrations below this figure it was necessary to increase the concentration of sodium succinate to 15mM to permit accurate measurement of oxygen uptake to be made. Glucose, KCl and ADP had no effect on the respiration rate and were omitted from the medium. To improve stability, cells were isolated in calcium-free Locke's solution containing citrate and not isolated in mannitol as used by Ichihara et al but the additional components (39mM NaCl, 1.4mM KCl, 0.25mM $NaHCO_3$, 1.37mM glucose and 6.7mM sodium citrate) present in the final medium did not alter the respiration rate. The pH of the incubation medium was 7.2 and it may be seen in figure 6 that respiration rate did not vary over the range pH 7.0 to 7.4. However, at pH's outside this range the respiration rate was reduced. In the absence of magnesium or phosphate the respiration rate was reduced to approximately one-half its value while almost all respiration was abolished if

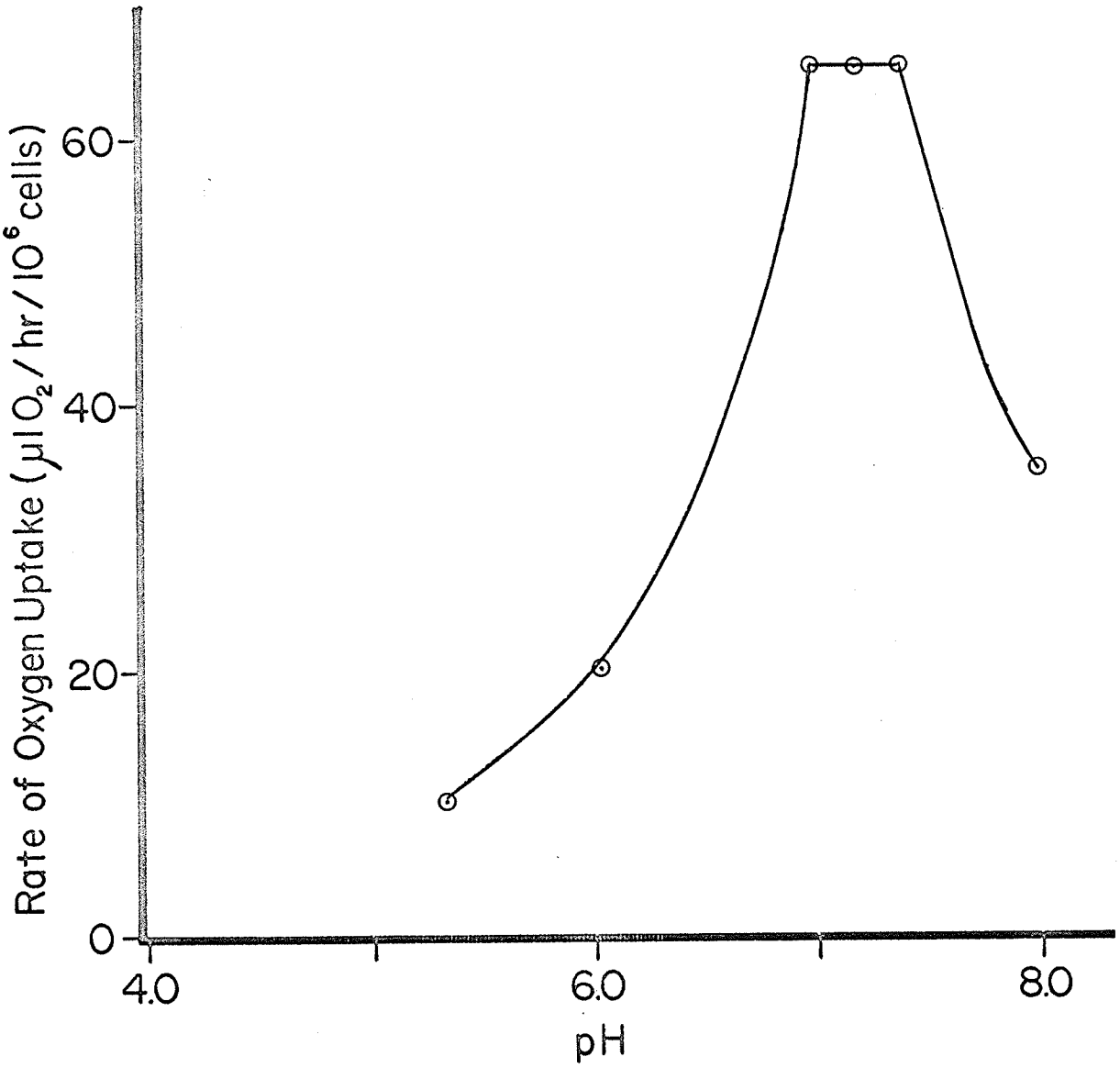


Figure 6. Effect of pH on control respiration rate.

succinate was omitted (table 8). Mannitol was retained in the medium to maintain the osmolarity. The concentrations of the components were varied one at a time. The optimal magnesium concentration was 1mM (figure 7b). Higher concentrations had no further effect. Varying the concentrations of phosphate over the range 10 to 40mM did not alter the respiration rate. When succinate concentration was varied no maximal rate was reached (figure 7a).

The final incubation medium contained 300mM mannitol, 20mM sodium phosphate pH 7.2, 15mM sodium succinate pH 7.2, 1mM $MgCl_2$, isolated cells, the components from the cell suspension and deionized water, and was called the basic incubation medium. The mean respiration rate of cells in the basic incubation medium from thirty different isolations was found to be 60.4 ± 0.4 $\mu l O_2$/hr/10^6 cells (s.d.) and was called the control respiration rate. This rate was maintained for sixty minutes and then declined slowly.

From table 5, it may be observed that 10^6 isolated rat liver cells is equivalent to 2mg dry weight of cells or 0.13mg of cellular nitrogen. Thus, 60 $\mu l O_2$/hr/10^6 cells is equivalent to 30 $\mu l O_2$/hr/mg dry weight of cells or 460 $\mu l O_2$/hr/mg cellular nitrogen or 660 $\mu g O_2$/hr/mg cellular nitrogen. Using the data in figure 7a, the respiration rate for isolated liver cells in the present study, values in brackets, are similar to values reported by: a) Ichihara,

TABLE 8.

Requirements for Control Respiration Rate

Omission from Basic Medium	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)
none	61
succinate	7
magnesium	28
phosphate	30
mannitol	54

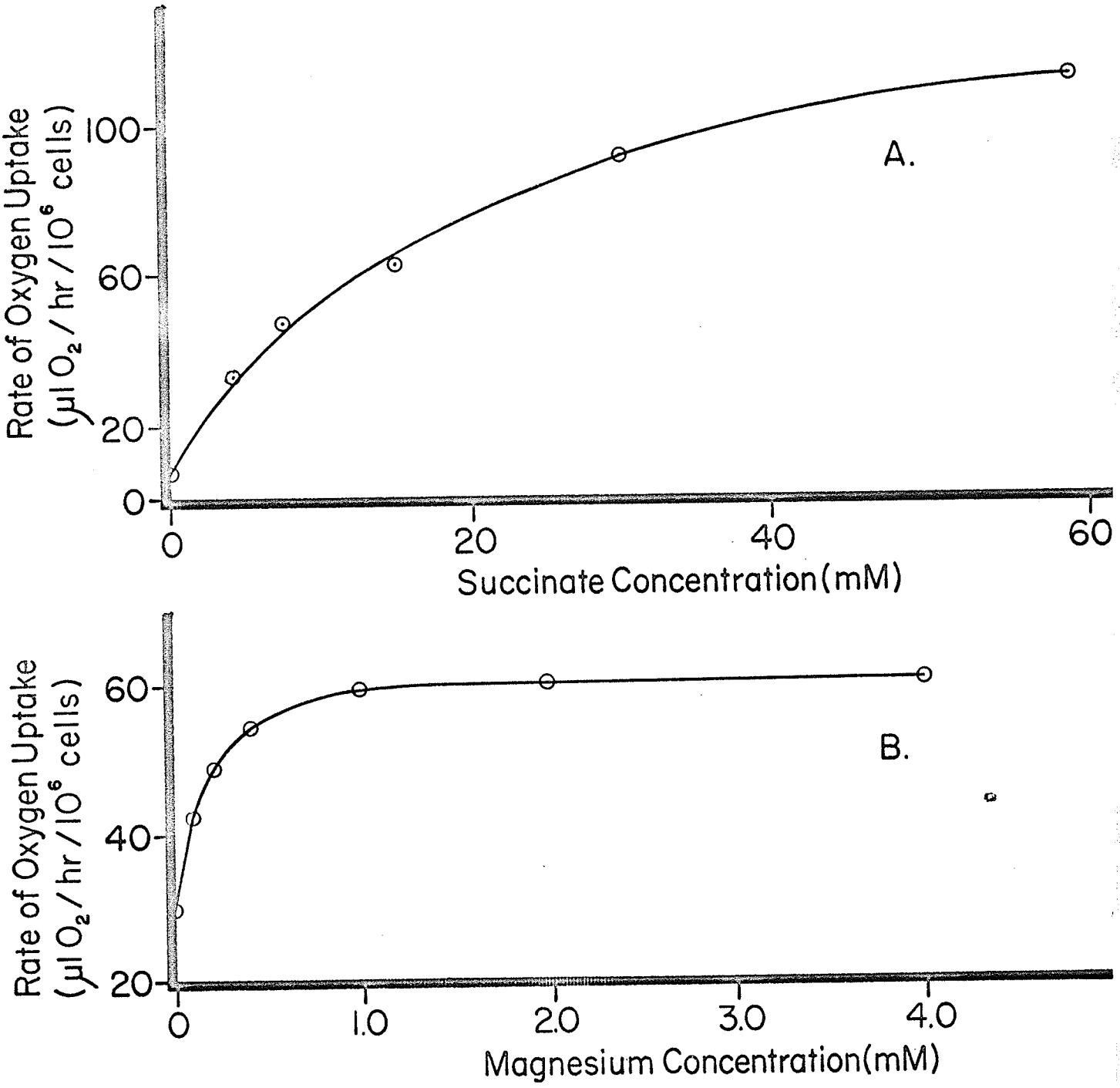


Figure 7. Effect of varying the concentration of succinate and magnesium on the control respiration rate.

Tanioka and Takeda (1965), 21.5 $\mu l O_2$/hr/mg dry weight of cells (11.5 $\mu l O_2$/hr/mg dry weight of cells), at a concentration of 2.5mM succinate; b) Green (1965), 123 $\mu l O_2$/hr/10^6cells (75 $\mu l O_2$/hr/10^6cells), at a concentration of 21mM succinate; c) Kalant and Young (1957), 390 $\mu l O_2$/hr/mg cellular nitrogen (729 $\mu l O_2$/hr/mg cellular nitrogen), at a concentration of 33mM succinate. Aside from the present work, effects of PTH on isolated cells have only been reported by Arnaud and Rasmussen (1964) using a kidney tubular preparation. They obtained an endogenous respiration rate of 67 $\mu g O_2$/hr/mg cellular nitrogen which is comparable to the endogenous rate of 77 $\mu g O_2$/hr/mg cellular nitrogen for isolated liver cells in the present study. The close similarity of these values is in agreement with the report of Kalant and Young (1957) that there is no difference between the respiration rates of isolated liver and kidney cells.

Effect of Oligomycin, ATP and PTH on Respiration Rate of Isolated Cells

Oligomycin in the concentration range of 3 to 8μg/ml has been shown to inhibit respiration of isolated rat liver and kidney mitochondria (Fang and Rasmussen, 1964) and of isolated rat renal tubular cells (Arnaud and Rasmussen, 1964). In contrast, in the present study, 3.85μg/ml oligomycin had no effect on the respiration rate of isolated rat liver cells. However, when 0.5mM ATP, as well as oligomycin, was present

the respiration rate was markedly reduced (figure 8). The mean value from twenty-five determinations was 29.7 ± 0.6 $\mu l O_2/hr/10^6$ cells (s.d.). Similar data was recently reported by Sallis et al (1966). The respiration rate of rat liver mitochondria was reduced from 80 $\mu l O_2/hr/mg$ nitrogen to 70 $\mu l O_2/hr/mg$ nitrogen by 3.3μg/ml oligomycin and was further reduced to 55 $\mu l O_2/hr/mg$ nitrogen when 0.67mM ATP was also added.

In the absence of oligomycin and ATP, Sallis et al (1966) also reported that 67μg/ml PTH increased the respiration rate from 80 $\mu l O_2/hr/mg$ nitrogen to 159 $\mu l O_2/hr/mg$ nitrogen, whereas in the presence of oligomycin and ATP the the rate of 55 $\mu l O_2/hr/mg$ nitrogen was increased to 242 $\mu l O_2/hr/mg$ nitrogen. This is in contrast to the present work on isolated liver cells where 8.5μg/ml PTH had no effect on the control respiration rate and, in the presence of oligomycin and ATP, only increased the respiration rate back to the control level (figure 8). In the presence of oligomycin, ATP and PTH, the mean rate from twenty-five determinations was 61.0 ± 0.5 $\mu l O_2/hr/10^6$ cells (s.d.), which is not significantly different from the control respiration rate of 60.4 ± 0.4 $\mu l O_2/hr/10^6$ cells (s.d.). Similarly, Arnaud and Rasmussen (1964) found that 80μg/ml PTH had no effect on the endogenous respiration rate of kidney tubular cells unless the rate was first reduced. However, their work differed in that oligomycin alone reduced the endogenous

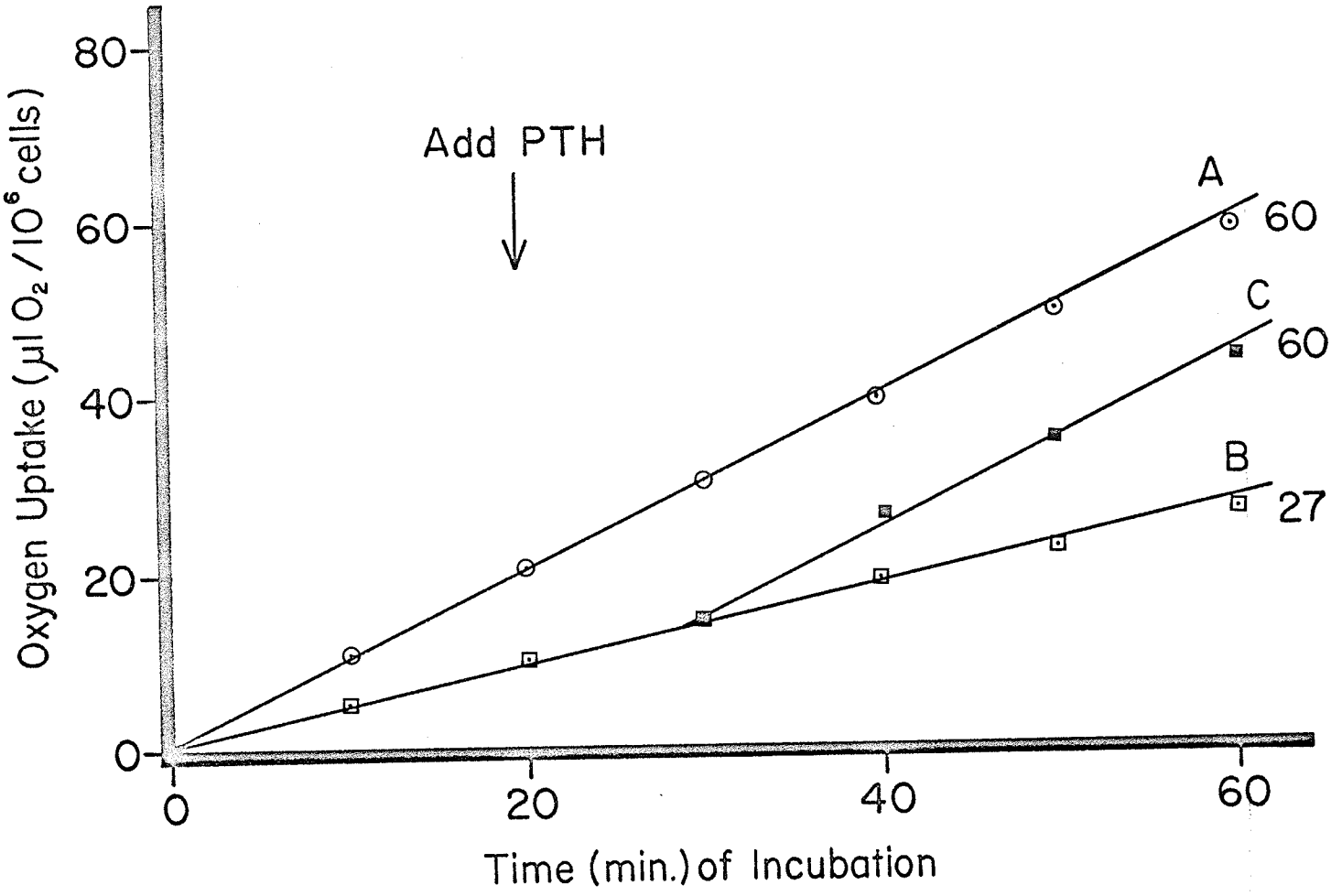


Figure 8. Effect of oligomycin, ATP and PTH on oxygen uptake.

Oxygen uptake of isolated cells in the basic incubation medium A. without addition. B. with 3.85µg/ml oligomycin and 0.5mM ATP in addition. C. as in B. with addition of 8.5µg/ml PTH from the sidearm after twenty minutes of incubation.

respiration rate from 67 $\mu g O_2$/hr/mg nitrogen to 10 $\mu g O_2$/hr/mg nitrogen and the hormone only increased the reduced rate to 14 $\mu g O_2$/hr/mg nitrogen.

Concentrations of ATP from 0.05 to 1.0mM in the presence of 3.85µg/ml oligomycin (table 9) and of oligomycin from 0.44 to 14.00µg/ml in the presence of 0.5mM ATP (table 10) reduced the respiration rate to the same extent. In the presence of oligomycin and ATP, PTH produced an increase in respiration over the pH range from 6.05 to 7.4 but not at pH 8.0 (table 11). The respiration rate was not fully restored to the control level at PTH concentrations less than 6.8µg/ml but bore a linear relationship to concentration of hormone below this value (figure 9). The concentrations of ATP, oligomycin and PTH chosen and used throughout the present work were 0.5mM, 3.85µg/ml and 8.5µg/ml respectively. If succinate, magnesium or phosphate were omitted from the medium, the respiration rate was reduced and the addition of oligomycin and ATP was without effect (table 12). However, if PTH was also added, the respiration rate was increased in the absence of phosphate but not in the absence of succinate or magnesium (table 13).

Effect of Replacing Medium Components on Respiration Rate of Isolated Cells

Phosphate was not an absolute requirement for the control respiration rate although respiration was reduced

TABLE 9.

Effect of ATP Concentration on Respiration Rate in the Presence of Oligomycin

ATP Concentration (mM) in Basic Medium Containing Oligomycin	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)	
	PTH Absent	PTH Present
0	60	60
0.05	30	56
0.10	27	60
0.25	30	60
0.50	30	62
1.00	30	60

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of oligomycin and PTH were 3.85µg/ml and 8.5µg/ml respectively.

TABLE 10.

Effect of Oligomycin Concentration on Respiration Rate in the Presence of ATP

Oligomycin Concentration (µg/ml) in Basic Medium Containing ATP	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)	
	PTH Absent	PTH Present
0	60	60
0.44	25	60
0.88	25	59
1.75	25	57
3.50	25	60
7.00	25	58
14.00	25	58

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of ATP and PTH were 0.5mM and 8.5µg/ml respectively.

TABLE 11.

Effect of pH on Respiration Rate in the Presence of Oligomycin, ATP and PTH

pH of Basic Medium Containing Oligomycin and ATP	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)	
	PTH Absent	PTH Present
6.05	25	55
7.00	30	66
7.20	30	68
7.40	30	68
8.00	39	39

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of oligomycin, ATP and PTH were 3.85µg/ml, 0.5mM and 8.5µg/ml respectively.

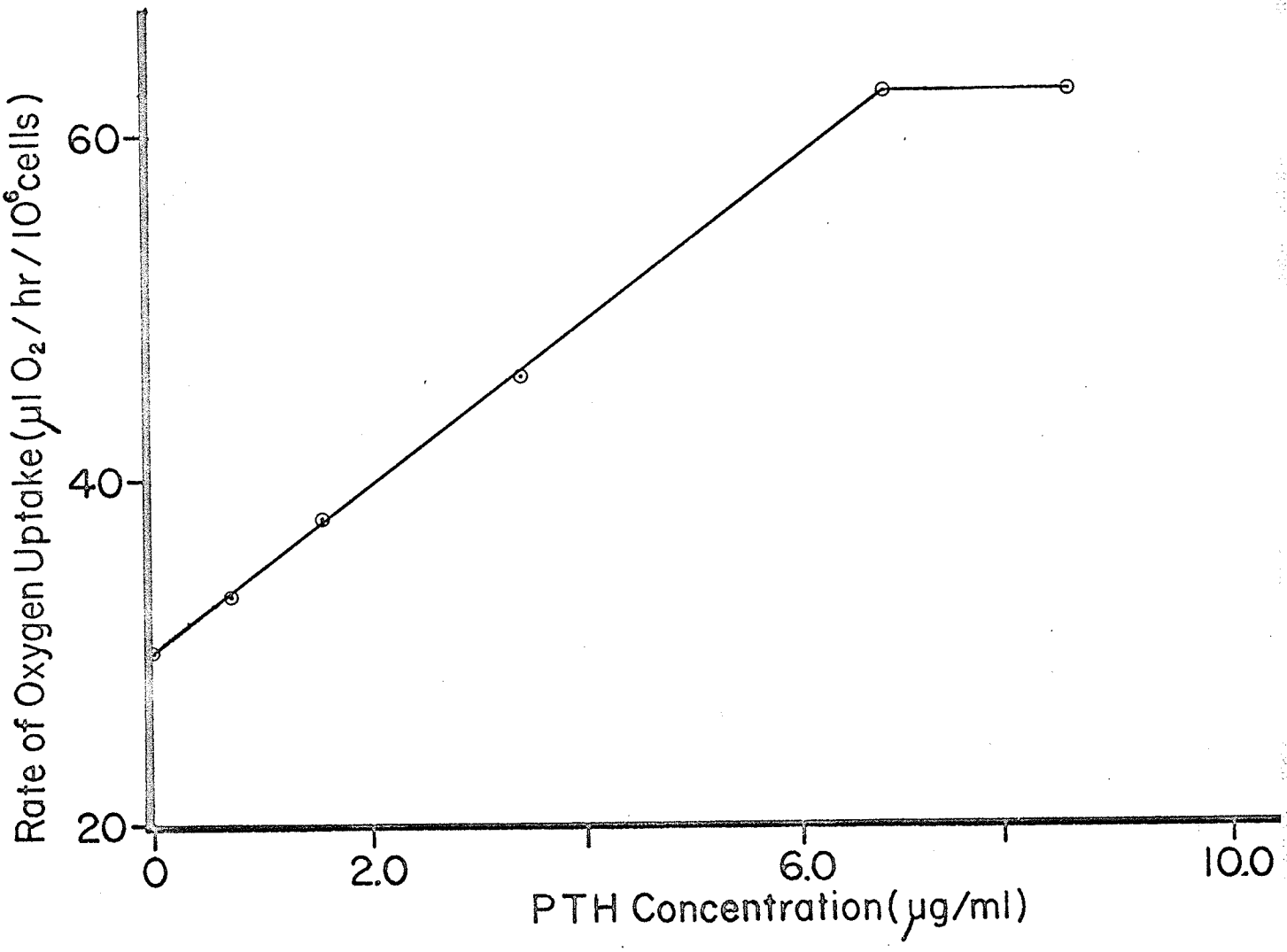


Figure 9. Effect of PTH concentration on respiration rate in the presence of oligomycin and ATP.

PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of oligomycin and ATP were 3.85µg/ml and 0.5mM ATP respectively.

TABLE 12.

Effect of Oligomycin and ATP on Respiration Rate in Deficient Media

Omission from Basic Medium	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)	
	Oligomycin and ATP Absent	Oligomycin and ATP Present
none	61	30
succinate	7	10
magnesium	28	30
phosphate	30	27
mannitol	54	29

Note: The final concentrations of oligomycin and ATP were 3.85μg/ml and 0.5mM respectively.

TABLE 13.

Effect of PTH on Respiration Rate in Deficient Media Containing Oligomycin and ATP

Omission from Basic Medium Containing Oligomycin and ATP	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)	
	PTH Absent	PTH Present
none	30	60
succinate	10	10
magnesium	30	30
phosphate	27	60
mannitol	29	63

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of oligomycin, ATP and PTH were 3.85µg/ml, 0.5mM and 8.5µg/ml respectively.

in its absence. It could be fully replaced by arsenate but only partially by sulfate (table 14).

Kalant and Young (1957) reported that isolated rat liver cells had a very low rate of endogenous respiration. There was also little oxidation of added respiratory substrates other than succinate which was oxidized at a higher rate than in tissue slices. Ichihara, Tanioka and Takeda (1965) demonstrated that addition of ADP to a medium containing respiratory substrates other than succinate increased the respiration rate but not to the value obtained with succinate. Similar data is presented in table 15 where in the absence of respiratory substrate the rate was only 10 $\mu l O_2/hr/10^6$ cells and respiratory substrates other than succinate did not increase the endogenous rate unless ADP or NAD were present. In the presence of both ADP and NAD the respiration rate with glutamate was further increased but was still lower than the value obtained with succinate. This requirement for ADP and NAD is consistent with the finding of Gibbons and Rienits (1961) that these cofactors leak out from dispersed rat liver cells upon isolation. The more rapid oxidation of succinate is probably due to its being FAD rather than NAD dependent and also to a greater mitochondrial permeability to succinate. Lynn and Brown (1966) have reported that respiratory substrates and other organic ions are taken up <u>in vitro</u> by rat liver mitochondria and that succinate is accumulated to a greater extent than the

TABLE 14.

Effect of Replacement of Phosphate on Respiration Rate

Addition to Basic Medium Deficient in Phosphate	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)
none	30
phosphate	58
sulfate	41
arsenate	60

Note: The final concentration of phosphate, sulfate or arsenate was 20mM.

TABLE 15.

Effect of Respiratory Substrates on Respiration Rate

Additions to Basic Medium Deficient in Respiratory Substrate	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)		
	Oligomycin, ATP and PTH Absent	Oligomycin and ATP Present	Oligomycin, ATP and PTH Present
none	10		
succinate	64		
citrate	11		
fumarate	11		
malate	11		
glutamate	10	10	10
α-ketoglutarate	9		
glutamate and ADP	30		
glutamate and NAD	29		
glutamate, ADP and NAD	41	22	45
α-ketoglutarate, ADP and NAD	40	24	44

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of added components were 3.85μg/ml oligomycin, 0.5mM ATP, 8.5μg/ml PTH, 15mM respiratory substrates, 1.5mM ADP and 0.8mM NAD.

other Krebs' cycle anions. In the absence of ADP and NAD, PTH had no effect on the oxidation of respiratory substrates other than succinate, even in the presence of oligomycin and ATP. When the two cofactors were added however, the respiration rate was reduced by oligomycin and ATP and restored upon addition of PTH.

The ability of a number of cations of varying valency to replace magnesium was tested by addition to the basic incubation medium deficient in magnesium (table 16). Sodium, barium and ferric ions were without effect whereas manganese could completely replace magnesium. The reduction in respiration rate by oligomycin and ATP and the subsequent increase on addition of PTH were identical whether magnesium or manganese was present. Replacement of magnesium by calcium resulted in a 50% increase in the respiration rate and this high rate was unchanged in the presence of oligomycin and ATP or upon subsequent addition of PTH.

Effect of Metabolic Inhibitors on Respiration Rate of Isolated Cells

In a previous section, it was shown that oligomycin alone did not reduce the respiration rate but did in combination with ATP (table 9). Results obtained with certain other metabolic inhibitors of oxidative phosphorylation are shown in table 17. Two known inhibitors of electron transport, sodium cyanide and antimycin A, reduced the respiration rate

TABLE 16.

Effect of Cations on Respiration Rate

Addition to Basic Medium Deficient in Magnesium	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)		
	Oligomycin, ATP and PTH Absent	Oligomycin and ATP Present	Oligomycin, ATP and PTH Present
none	30		
NaCl	28		
$BaCl_2$	32		
$FeCl_3$	34		
$MgCl_2$	59	30	60
$MnCl_2$	60	27	60
$CaCl_2$	93	93	93

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of added components were 3.85µg/ml oligomycin, 0.5mM ATP, 8.5µg/ml PTH and 1mM cation chlorides.

TABLE 17.

Effect of Metabolic Inhibitors on Respiration Rate

Additions to Basic Medium	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)		
	PTH and DNP Absent	PTH Present	DNP Present
none	60		60
oligomycin	60		
oligomycin and ATP	25	58	
sodium cyanide	30	30	30
antimycin A	6	6	6
gramicidin	60		
ouabain	62		
ouabain, oligomycin and ATP	25	58	

Note: When present, DNP was added at the beginning of the incubation while PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of added components were 3.85µg/ml oligomycin, 1×10^{-3}M sodium cyanide, 1.3×10^{-5}M antimycin A, 7×10^{-5}M gramicidin, 5×10^{-4}M DNP, 0.15mM ouabain, 0.5mM ATP and 8.5µg/ml PTH.

and the further addition of PTH or DNP had no effect. Gramicidin and DNP, at concentrations that produce uncoupling of oxidative phosphorylation in tissues, did not increase the respiration rate. In the presence of ouabain, an inhibitor of cytoplasmic but not mitochondrial ATPase activity (Blond and Whittam, 1964), the control rate, the reduced rate in the presence of oligomycin and ATP and the subsequent increase in respiration upon addition of PTH, were all unchanged.

Effect of Nucleotides on Respiration Rate of Isolated Cells

Respiration was reduced to the same extent by oligomycin in combination with ADP or AMP as with ATP, and to a lesser extent in combination with adenosine or dATP (table 18). In all cases, the rate returned to the control value on addition of PTH. Sallis and Deluca (1966) found that, if albumin was present, oligomycin was a more effective inhibitor of respiration in isolated rat liver mitochondria. However, as shown in table 18, oligomycin was without effect on isolated rat liver cells in the presence as in the absence of albumin. Previously, it was shown that ATP alone did not alter the respiration rate but in combination with oligomycin the rate was lowered (table 10). It may be seen in table 18 that ADP, AMP or adenosine also did not produce any change in the rate if oligomycin was not added. In contrast to this requirement for oligomycin in the case of the adenosine

TABLE 18.

Effect of Nucleotides on Respiration Rate

Additions to Basic Medium	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)	
	PTH Absent	PTH Present
none	59	59
oligomycin and ATP	24	60
oligomycin and ADP	27	59
oligomycin and AMP	29	60
oligomycin and adenosine	40	59
oligomycin and dATP	53	58
oligomycin and albumin	60	
ATP	60	
ADP	60	
AMP	60	
adenosine	60	
GTP	24	60
UTP	27	64
CTP	24	60
ITP	27	64

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of added components were 3.85µg/ml oligomycin, 0.5mM nucleotides and nucleosides, 8.5µg/ml PTH and 0.6mg/ml albumin.

nucleotides, other nucleoside triphosphates, GTP, UTP, CTP or ITP, reduced the rate in its absence. On further addition of PTH the rate returned to the control value.

Replacement of the triphosphates was tried in the inosine series (table 19). While inosine was without effect, results with IDP or IMP were identical to those with ITP. The rate was not lowered if, as well as ITP, an adenosine nucleotide was present from the beginning of incubation and and was found to return to the control value if the adenosine nucleotide was added from the sidearm. If however, oligomycin was present as well as ITP and ADP the rate was lowered.

A possible explanation for the fact that AMP, ADP and ATP were equally effective could be a ready interconversion between these nucleotides in the cells. Such interconversion, resulting from oxidative phosphorylation and ATPase and adenylate kinase activities, occurs in isolated rat liver mitochondria (Connelly and Hallstrom, 1966). Similar conclusions about isolated rat liver cells may be drawn from the results shown in table 20. Due to oxidative phosphorylation, most of the nucleotide phosphate isolated when either ATP, ADP or AMP was added to the incubation medium was ATP. ATPase and adenylate kinase activities were also present because, if oxidative phosphorylation was suppressed by the omission of respiratory substrate, ADP as well as ATP was isolated when ATP was added, and ADP as well as AMP when AMP was added.

TABLE 19.

Effect of Inosine Nucleotides on Respiration Rate

Additions to Basic Medium	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)	
	PTH Absent	PTH Present
none	60	60
ITP	30	60
IDP	28	60
IMP	32	60
inosine	60	60
ITP and ATP	62	
ITP and ADP	64	
ITP and AMP	64	
ITP, ADP and oligomycin	32	

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of added components were 3.85µg/ml oligomycin, 0.5mM nucleotides and nucleosides and 8.5µg/ml PTH.

TABLE 20.

Interconversion of Adenosine Nucleotides by Isolated Cells

Medium Used	Nucleotide Addition	Concentration of Adenosine Nucleotides After Thirty Minutes of Incubation (mM)		
		ATP	ADP	AMP
basic medium	none	0	0	0
	ATP	5.3	0	0
	ADP	4.5	0.3	0
	AMP	4.1	0.7	0
	ITP	0	0	0
basic medium deficient in succinate	ATP	3.2	1.5	0
	AMP	0	2.5	2.1

Note: The initial concentration of all nucleotides was 5.0mM.

Effect of Basic Proteins and Uncouplers of Oxidative Phosphorylation on Respiration Rate of Isolated Cells

Aurbach, Houston and Potts (1965) demonstrated that the non-hormonal basic proteins, polylysine and protamine sulfate, were able to stimulate, as did PTH, respiration, ion transport and ATPase activity in mitochondria isolated from liver or kidney. They therefore questioned the specificity of these in vitro PTH effects and suggested that they could be a result of the basic nature of the hormone. In line with these results, protamine sulfate and two polylysine preparations of molecular weight 42,000 and 4,000 could replace PTH; increasing the respiration rate of isolated rat liver cells after this had been lowered by either ITP or oligomycin and ATP (table 21). Also, two known uncouplers of oxidative phosphorylation, DNP and gramicidin, were as effective as PTH or the basic proteins (table 22).

The respiration rate in the absence of phosphate was low and was not affected by the addition of oligomycin and ATP, polylysine, DNP or PTH (table 23). However, in a phosphate-deficient medium containing oligomycin and ATP, PTH stimulated respiration specifically, DNP and polylysine being ineffective. Although the respiration rate was lowered to the same extent in a magnesium-deficient as in a phosphate-deficient medium, it was not altered by the addition of polylysine, DNP or PTH whether oligomycin and ATP were present or not. Thus, an

TABLE 21.

Effect of Basic Proteins on Respiration Rate

Additions to Basic Medium	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)				
	PTH, Protamine Sulfate and Polylysine Absent	PTH Present	Protamine Sulfate Present	Polylysine I Present	Polylysine II Present
oligomycin and ATP	30	66	64	64	64
ITP	34	64	64	64	64

Note: PTH and other basic proteins were added from the sidearm after twenty minutes of incubation. The molecular weight of polylysine I was 42,000 and of polylysine II, 4,000. The final concentrations of added components were 3.85μg/ml oligomycin, 0.5mM nucleotide triphosphates, 8.5μg/ml PTH and 10μg/ml non-hormonal basic proteins.

TABLE 22.

Effect of Uncouplers of Oxidative Phosphorylation on Respiration Rate

Addition to Basic Medium Containing Oligomycin and ATP	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)
none	33
PTH	60
DNP	60
gramicidin	60

Note: PTH, DNP and gramicidin were present from the beginning of the incubation. The final concentrations of added components were 3.85μg/ml oligomycin, 2×10^{-5}M DNP, 7×10^{-5}M gramicidin, 0.5mM ATP and 8.5μg/ml PTH.

TABLE 23.

Effect of PTH, DNP and Polylysine on Respiration Rate in Phosphate- and in Magnesium-Deficient Media

Medium Used	Additions	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)		
		PTH and Polylysine Absent	PTH Present	Polylysine Present
basic medium deficient in phosphate	phosphate	62		
	none	23	23	23
	oligomycin and ATP	24	60	26
	DNP	27		
	oligomycin, ATP and DNP	27		
basic medium deficient in magnesium	magnesium	62		
	none	23	23	23
	oligomycin and ATP	24	24	23
	DNP	26		
	oligomycin, ATP and DNP	27		

Note: PTH and polylysine of molecular weight 42,000 were added from the sidearm after twenty minutes of incubation. The final concentrations of added components were 3.85µg/ml oligomycin, 5×10^{-4}M DNP, 0.5mM ATP, 8.5µg/ml PTH and 10µg/ml polylysine.

increase in respiration occurred only in the phosphate-deficient medium containing oligomycin and ATP in combination with PTH. By adding oligomycin or ATP separately, it was found that ATP was the necessary requirement for the hormone stimulation (table 24). ATP could be completely replaced by the nucleotides ADP, AMP, GTP, UTP, CTP and ITP but not by the nucleoside adenosine. Combinations of DNP or polylysine with either oligomycin or ATP produced no change in respiration rate in the phosphate-deficient medium, just as in the case when oligomycin and ATP were present together.

Effect of PTH Treated in Various Ways on Respiration Rate of Isolated Cells

Oxidation of PTH with hydrogen peroxide results in the loss of both the calcium mobilizing and phosphaturic activities *in vivo* (Tashjian, Ontjes and Munson, 1964). Similar results were obtained with PTH isolated for the present study (figure 10). After parathyroidectomy, the calcium level of rat serum fell continually with time even after the administration subdermally of PTH heated to 100°C for one hour or of PTH oxidized with hydrogen peroxide. When oxidized PTH which had been reduced with cysteine-HCl, untreated PTH or parathyroid extract (Lilly, U.S.P.) was administered, the calcium level rose to a maximum comparable to the level prior to parathyroidectomy. Because PTH must be in the reduced form to act *in vivo*, it was important to

TABLE 24.

Effect of Nucleotides on Respiration Rate in Phosphate-Deficient Medium

Additions to Basic Medium Deficient in Phosphate	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)	
	PTH Absent	PTH Present
phosphate	60	
none	30	
oligomycin and ATP	27	60
oligomycin	29	29
ATP	32	60
ADP	30	60
AMP	27	60
adenosine	30	30
GTP	30	60
UTP	30	60
CTP	30	60
ITP	30	63

Note: PTH was added from the sidearm after twenty minutes of incubation. The final concentrations of added components were 3.85µg/ml oligomycin, 0.5mM nucleotides and nucleosides and 8.5µg/ml PTH.

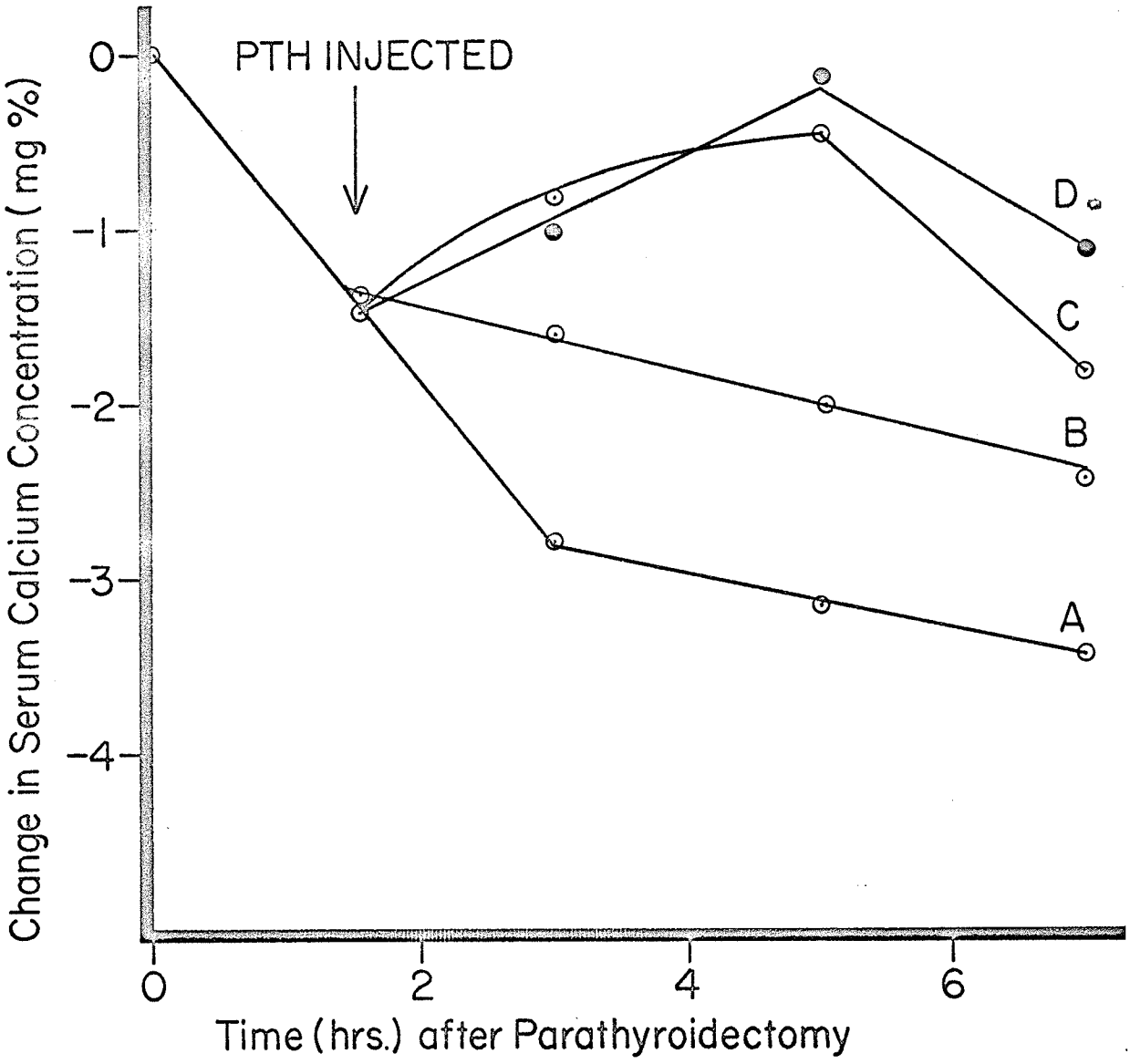


Figure 10. Effect upon injection of PTH treated in various ways on the serum calcium of parathyroidectomized rats.

8.5μg of: (A) heated PTH, (B) oxidized PTH, (C) oxidized and reduced PTH and 50 units of: (D) parathyroid extract (Lilly, U.S.P.) were injected at one and one-half hours after parathyroidectomy.

determine the effect of oxidation of the hormone on its ability to increase respiration in isolated rat liver cells. In the presence of oligomycin and ATP, heated or oxidized PTH was inactive, whereas oxidized and reduced PTH was as effective as untreated hormone (table 25). Heated or oxidized PTH was also inactive in a phosphate-deficient medium containing ATP, whereas oxidized and reduced PTH increased the reduced rate but was not, in this case, fully as effective as untreated hormone. Thus, the <u>in vitro</u> effect of PTH on cellular respiration was as dependent on the oxidation state as was its <u>in vivo</u> action on serum calcium.

TABLE 25

Effect of PTH Treated in Various Ways on Respiration Rate

Medium Used	Rate of Oxygen Uptake ($\mu l O_2/hr/10^6$ cells)				
	PTH Absent	PTH Present	Oxidized PTH Present	Heated PTH Present	Oxidized and Reduced PTH Present
basic medium containing oligomycin and ATP	30	65	30	30	65
basic medium deficient in phosphate and containing ATP	34	66	34	34	42

Note: All PTH preparations were added from the sidearm after twenty minutes of incubation. The final concentrations of added components were 3.85µg/ml oligomycin, 0.5mM ATP and 8.5µg/ml PTH.

DISCUSSION OF RESULTS

A single preparation of hormone, isolated by gel filtration on Sephadex G-100, was used in all experiments. The preparation was not completely homogeneous on electrophoresis in polyacrylamide gel but, when assayed in parathyroidectomized rats, was found to have a potency of approximately 3,000 units/mg, an activity similar to that reported by Aurbach and Potts (1964). It also stimulated phosphate uptake in isolated rat liver mitochondria (table 2), a property found by Sallis et al, 1963 for PTH prepared in a similar manner.

Although no direct measurements were made, it is reasonable to assume that cofactors and enzymes were lost from the rat liver cells upon isolation. The fact that glucose could not support respiration indicates that necessary glycolytic enzymes or cofactors were missing. Also, the cells were isolated by a method quite similar to those which other investigators (see History of the Literature) have shown involved such loss as a result of an alteration in cell wall permeability. When lissamine green, commonly used as an indicator of cell permeability (Kay, 1965), was added to the rat liver cells, 100% took up the dye. It is quite possible, therefore, that the larger components of the incubation medium, including oligomycin, nucleotides and even PTH, could penetrate the cells. Tobin and Slater (1965) reported that oligomycin reached the mitochondria even in tissue slice

preparations. The determination of protein uptake into cells (Perkin et al, 1967, Caulfield, 1963 and Ryser, 1963) has been facilitated by development of methods for labelling protein molecules with fluorescein (Rinderknecht, 1962), ferritin (Singer and McLean, 1963) or ^{131}I (Hunter and Greenwood, 1962) which in turn may be detected by fluoresence microscopy, electron microscopy and radiotracer methods. Using such methods, Ryser and Hancock (1965) demonstrated that basic proteins (histones and polyamino acids such as polylysine) are readily taken up into the cytoplasm of sarcoma cells. Because of the increased permeability of the isolated cells and the basic nature of PTH, it is probable that the hormone can enter the cells and act directly on the mitochondrion. PTH labelled with ^{131}I has already been prepared (Berson et al, 1963) and there is no reason why the hormone could not also be labelled with fluorescein or ferritin. It may, therefore, be possible to determine if cellular penetration by the hormone occurs, although the demonstration of binding to mitochondria within cells may be outside the limits of the methods, for Pastan et al (1966) have reported that even radioiodinated hormones are not wholly satisfactory for the intracellular localization of the biologically significant binding sites because of the low uptake of labelled hormone. However, if the hormone could be shown to penetrate cells, this, along with the demonstration that isolated rat liver mitochondria could bind ^{131}I-PTH,

would provide strong support for a hormonal action directly upon the mitochondria.

The control respiration rate was unaffected by the addition of oligomycin (tables 9 and 17). Because oligomycin readily enters cells, respiration in the control state must be "uncoupled". Currie and Gregg (1965) and Tobin and Slater (1965) presented evidence for the existence of uncoupled respiration in a variety of tissues, including liver, in which oligomycin only inhibited, to a small degree, the respiration in slice preparations. The energy could be utilized for ion transport into mitochondria because optimal respiration of the cells occurred over a pH range of 7.0 to 7.4 (figure 6), identical to that found by Rasmussen (1966) for respiratory-supported magnesium phosphate uptake into isolated rat liver mitochondria. Also, the ability to replace phosphate by sulfate or arsenate (table 14) and magnesium by manganese or calcium (table 16) is similar to that reported by Fang and Rasmussen (1964) for isolated rat liver mitochondria in which respiratory-supported translocation of phosphate, sulfate or arsenate occurs if magnesium or manganese is present.

In contrast to the lack of effect of oligomycin alone, the addition of any adenosine nucleotide with oligomycin resulted in a reduced respiration rate (table 18) indicating "coupling". The adenosine nucleotide alone causes the coupling because, after the addition of AMP, ADP or ATP, most of the

nucleotide isolated was ATP (table 20). No change in respiration accompanied the coupling perhaps because of the ready availability of the phosphate acceptor, ADP, added directly or produced by nucleotide interconversion (table 20) from either ATP by the action of an ATPase or from AMP by the action of an adenylate kinase. There would have to be sufficient endogenous ATP initially present in the isolated cells for the adenylate kinase action on AMP. However, this would not be necessary if the recent suggestion of Ozawa (1966), that AMP and not ADP be the first phosphoryl acceptor in oxidative phosphorylation, is correct.

Non-adenosine nucleotides, namely GTP, UTP, CTP, ITP, IDP or IMP, separately, reduced the cellular respiration rate (tables 18 and 19). When ADP was added to a medium containing ITP, respiration was increased and was now susceptible to inhibition by oligomycin (table 19). Therefore, these non-adenosine nucleotides, while able to couple respiration to ATP formation, were not functioning as phosphate acceptors. This lack of acceptor function in a cellular preparation is consistent with the demonstration by Low et al (1963) that GDP, UDP, CDP or IDP could not replace ADP as a phosphate acceptor in oxidative phosphorylation in isolated beef heart mitochondria though they could in submitochondrial particles. Pfaff et al (1965), in explanation of the results of Low et al (1963), suggested that, when submitochondrial particles are prepared by sonication, the oxidative phosphorylation

compartment is exposed to added nucleotides. On the other hand, in intact mitochondria, the enzymes of oxidative phosphorylation could be inaccessible to non-adenosine nucleotides while the transport of adenosine nucleotides is facilitated and controlled by a specific exchange enzyme.

Nucleosides or dATP in combination with oligomycin did not affect the respiration rate (tables 18 and 19). This indicates that a nucleotide containing a ribose moiety is necessary for coupling to occur. However, the number of phosphate moieties required is uncertain at present because of the ready interconversion of nucleotides. This uncertainty could possibly be removed by studying the interconversion of inosine nucleotides. If no such interconversion occurred, all nucleoside phosphates would be effective. If, however, interconversion of any or all of the nucleoside phosphates occurred, no definite conclusions could be made unless adequate inhibitors of the interconverting enzymes could be found.

Serum albumin is known to restore oxidative phosphorylation in isolated rat liver mitochondria uncoupled by various substituted phenols (Weinbach et al, 1963). Sallis and Deluca (1966), working with isolated rat liver mitochondria, demonstrated that the small reduction in respiration that occurred when oligomycin was added was more pronounced if ATP or albumin was present. Because ATP was equally as effective as albumin, they stated that the

nucleotide must also restore oxidative phosphorylation. This supports the present interpretation although serum albumin, in contrast to ATP, was not effective in isolated cells (table 18). The molecular weight of albumin is approximately 70,000. Due, perhaps to its large size or lack of basicity, albumin may not be able to penetrate the cells in sufficient quantity. Ryser and Hancock (1965) have shown that serum albumin is accumulated by sarcoma cells at a low rate, whereas basic proteins and polyamino acids are taken up at rates 3,000 times greater than serum albumin.

The respiration rate was not increased by PTH unless a nucleotide was present (tables 18, 19 and 24). It is probable that the nucleotide is required for the hormonal action in all situations but this requirement can only be conclusively demonstrated in the absence of phosphate (table 24). On the other hand, in the presence of phosphate, a hormonal effect could only be demonstrated after the respiration rate had been reduced by a non-adenosine nucleotide or an adenosine nucleotide plus oligomycin. Therefore it cannot, in this case, be established whether the nucleotide is only required for the reduction in respiration, or if it serves a dual function, being required for the hormonal stimulation of respiration as well. Since PTH is known to stimulate an ATPase action in rat liver mitochondria (Sallis and Deluca, 1964), it was initially thought that the PTH-induced increase in respiration in the cellular system could be due to such

stimulation, which would remove ATP and make ADP available for phosphorylation. However, the lack of effect of oligomycin, a known inhibitor of PTH-stimulated mitochondrial ATPase (Sallis and Deluca, 1964), and of ouabain, a known inhibitor of cytoplasmic ATPase activity (Blond and Whittam, 1964); the ability of the nucleotides ADP, AMP, GTP, UTP, CTP or ITP, inactive in Sallis and Deluca's ATPase study, to replace ATP (table 24) and the existence of a pH plateau of 6 to 7.4 (table 11) rather than the sharp pH optimum of 6.7 found by Sallis and Deluca, all make this explanation unlikely.

If, as seems probable, the nucleotide necessary to show a PTH effect on respiration is not supporting a PTH-stimulated ATPase, there must be some other reason, perhaps for coupling, for its required presence. Another possibility might be that the nucleotide aids in binding the hormone to mitochondria. Although no direct study of the binding of hormones to mitochondria has been made, a lot of work has been done on their binding to tissue slices. The paper by Pastan et al (1966) is the latest to report that a polypeptide hormone must first be bound to some cellular constituent. Using immunological techniques, they were able to work with physiological concentrations of thyroid stimulating hormone (TSH) and insulin. They demonstrated persistent effects on glucose-1-^{14}C oxidation in thyroid slices when these were exposed to TSH at $1^{\circ}C$, washed thoroughly in hormone-free medium and then incubated at $37^{\circ}C$. However, when the washed tissues were

subsequently exposed to anti-TSH serum or to trypsin, the persistent hormonal effect was abolished without damage to the cells. Similarly, the persistent effect of insulin on glucose-1-^{14}C incorporation into rat diaphragm could be abolished by anti-insulin serum.

The increase in cellular respiration by PTH must be due to an uncoupling of oxidative phosphorylation because the hormone increased the respiration rate in the presence of oligomycin but not in the presence of electron transport inhibitors (table 17) and because uncouplers of oxidative phosphorylation increased the respiration in a manner similar to PTH (table 22). In the absence of PTH, the addition of ADP to the basic medium led to ATP formation indicating coupling (table 20). More direct evidence that PTH acts as an uncoupler could possibly be obtained by determining if a reduction in ATP formation took place following addition of the hormone.

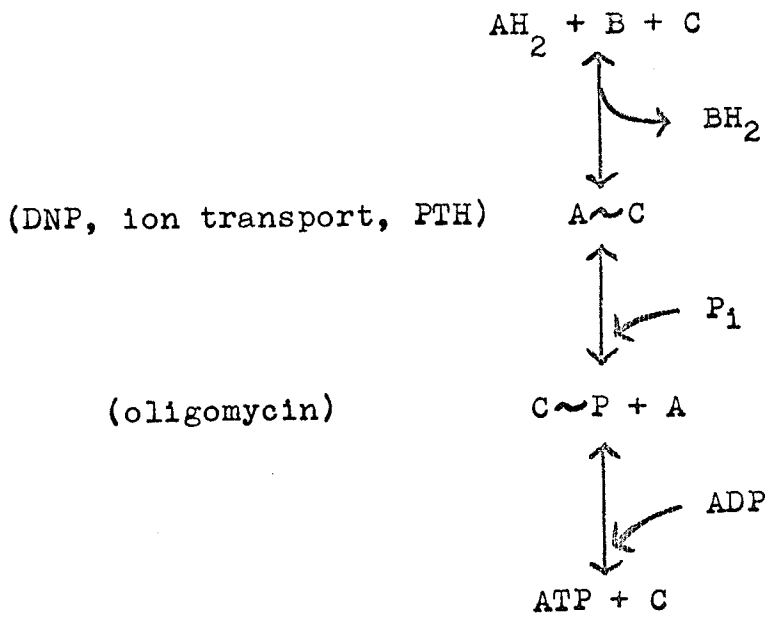
That PTH uncouples oxidative phosphorylation in isolated rat liver mitochondria (Fang et al, 1963, Sallis et al, 1964, Rasmussen, Sallis et al, 1964, Fang and Rasmussen, 1964, Utzumi et al, 1966, Rasmussen, 1966, Kimmich and Rasmussen, 1966 and Sallis and Deluca, 1966) has been established by showing that the hormone reduces the P/O ratio and increases respiration and ion transport in the presence of oligomycin but not in the presence of electron transport inhibitors. While the effects of PTH on isolated

mitochondria have received alot of attention, only one study has been reported on isolated cells. Arnaud and Rasmussen (1964) found that PTH increased the respiration rate of isolated rat kidney tubule cells in the presence of oligomycin.

The energy released in uncoupling by PTH in isolated cells may be redirected into ion transport. Energy not utilized for ion transport may be involved in swelling because PTH uncoupled oxidative phosphorylation in the absence of phosphate (table 24) as well as in its presence (table 18) and because it is known that PTH induces rapid and extensive mitochondrial swelling in the absence as well as in the presence of ion transport (Utzumi et al, 1966). Sallis and Deluca (1966), working with isolated rat liver mitochondria, found that PTH uncoupled in the presence as well as in the absence of ion translocation. In the present study, no phosphate uptake into cells incubated in the basic medium could be detected, although Ichihara, Tanioka and Takeda (1965), using a cell concentration ten times greater, have reported that isolated rat liver cells, similarly prepared and incubated, could take up phosphate ions. Because PTH stimulation of respiration in isolated mitochondria involves ion transport (Rasmussen, 1966), a phosphate uptake into isolated cells would be expected. Thus, it would be worthwhile to attempt to measure this using a greater cell concentration.

In the following diagram, PTH as an uncoupler has been

added, at point A~C, to the oxidative phosphorylation scheme already outlined in the History of the Literature. The choice



of this position is based largely on evidence from the work of others on isolated mitochondria. Sallis et al (1965) demonstrated that the PTH-dependent, respiratory supported, transport of magnesium phosphate into isolated rat liver mitochondria was not inhibited by oligomycin but was by DNP. The ATPase action by the hormone in isolated rat liver mitochondria was blocked by both oligomycin and DNP (Sallis and Deluca, 1964). If the ATPase action can be considered a reversal of oxidative phosphorylation, the site of action would then be at A~C. There is no evidence at present that this is not also the site of action of PTH in isolated cells.

In any attempt to explain how certain compounds can uncouple oxidative phosphorylation, the three dimensional

structure of the mitochondrion must be taken into account. One such hypothesis is that suggested by Crofts and Chappell (1965) who based it on the finding by Ohnishi and Ohnishi (1962) of a protein similar to the actomyosin of skeletal muscle in isolated rat liver mitochondria. They suggested that the uncoupling which accompanied the swelling produced by various agents was due to a physical separation of electron transport from the energy coupling process. If ATP was added, the swelling was reduced and oxidation was coupled to phosphorylation once more. Crofts and Chappell suggested that the actomyosin-like protein in the mitochondria contracted upon addition of ATP as it does in muscle, thus bringing the respiratory chain once more into proximity with the energy coupling process. This hypothesis, although logical, is not adequate because, whereas Weinbach et al (1963) found that the reversal of mitochondrial swelling was ATP-specific, uncoupling, in the present study, could be reversed by nucleotides other than ATP. Therefore, unless contraction of the actomyosin-like protein in mitochondria in isolated cells is facilitated by compounds other than ATP, a more appropriate hypothesis, such as that of Weinbach and Garbus (1964), is necessary. They demonstrated that various substituted phenols which are able to uncouple oxidative phosphorylation bind to the protein portion of isolated rat liver mitochondria. Serum albumin is able to restore oxidative phosphorylation by binding to the uncoupling phenol and removing it from the

mitochondria. They suggested that uncoupling agents interacted with the mitochondrial proteins that participate in oxidative phosphorylation, thereby inducing conformational changes analogous to allosteric effects. These altered configurations would result in modified enzymes and therefore loss of coupling activity. Applying this hypothesis to the present cellular system, the nucleotides could couple by interacting with the mitochondrial proteins and inducing a conformational change in the enzyme(s) catalyzing the reactions at A~C. Reversal of these effects could occur upon subsequent addition of PTH, either by removal of the coupling agent or further enzyme conformational changes.

Aurbach et al (1965) questioned the physiological significance of the in vitro effects of PTH on mitochondria because these could be duplicated by several non-hormonal basic proteins, including two isolated from parathyroid glands. In support of this work of Aurbach et al, basic proteins (table 21) and uncouplers of oxidative phosphorylation (table 22) behaved in a manner similar to PTH in the presence of phosphate. However, in the absence of phosphate, only PTH uncoupled, basic proteins and DNP being ineffective (table 23). Thus, the uncoupling action of PTH cannot be identical with that of DNP. Nor, apparently, is it due solely to the basic nature of the hormone, though, as discussed earlier, this may well be necessary to ensure access of the hormone to the mitochondria. That the basic nature of the hormone is