THE UNIVERSITY OF MANITOBA

THE EFFECTS OF EXPERIMENTAL INFLAMMATION ON ADAPTIVE SYNTHESIS OF FATTY ACID SYNTHETASE IN RAT LIVER

by

JOHN MCNEILL LANGSTAFF

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ABSTRACT

Initial studies indicated that the variations in FAS levels observed with adaptive synthesis and other physiological conditions were due to variations in the rates of enzyme synthesis and degradation. Experimental inflammation, induced by subcutaneous injection of oil of turpentine, suppresses the increase in hepatic FAS activity observed in rats, which were starved and refed a fat-free diet, to an extent dependent on the time interval between fat-free feeding and inflammation and inflammation and sacrifice. The mechanism of suppression, by experimental inflammation, of the increase in FAS levels with adaptive synthesis, was investigated immunochemically. That the suppression resulted from changes in amount of hepatic FAS was shown by the observation that fatty acid synthetase preparations from inflamed and non-inflamed animals, exhibiting a wide variety of specific enzyme activities, had identical immunochemical equivalence points. The amounts of fatty acid synthetase, determined by radial immunodiffusion in gels containing anti-FAS serum, varied concomitantly with changes in enzymic activity regardless of the relative times of inflammation and the onset of adaptive synthesis. Inflammation was found to decrease the relative rate of FAS synthesis and increase its relative rate of degradation to an extent which largely accounts for the variations in enzyme levels. An observed decrease in the relative rate of FAS degradation during the initial stages of adaptive synthesis was also found. Isolation and translation of poly(A)containing-RNA indicates

the variations in enzyme synthesis are at least partially dependent upon the levels of FAS message present in the cell. Besides this evidence of transcriptional control, some indications of the existence of some form of translational controls also appeared.

Inflammation resulted in a marked reduction in the level of liver glycogen which was accompanied by an elevation of hepatic cyclic AMP. Serum insulin levels were not dramatically elevated during the first 48 h of refeeding but rose markedly thereafter. Inflammation, either alone or combined with fat-free feeding, resulted in increased serum glucose levels, followed by a similar pattern of increased serum insulin levels some 12 h later. Fat-free feeding did not affect serum cortisol levels, but inflammation resulted in a marked increase in serum cortisol within 12 h. These results indicate possible roles for cortisol and cyclic AMP in the suppression of the synthesis of hepatic FAS.

Refeeding a fat-free diet after 48 h of starvation resulted in a rapid increase in the hepatic levels of acetyl-CoA carboxylase and ATP-citrate lyase. Inflammation markedly suppresses the adaptive synthesis of ATP-citrate lyase while only slightly suppressing the adaptive synthesis of acetyl-CoA carboxylase. Starvation and refeeding resulted in little variation in total lipid and cholesterol levels. Inflammation caused a rapid elevation in both, possibly indicating an increased synthesis of membrane components. Triglyceride levels decreased with starvation and rose to higher than normal levels with refeeding. This increase was suppressed by experimental inflammation.

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ABBREVIATIONS

A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
DTT	dithiothreitol
ER	endoplasmic reticulum
FAS	fatty acid synthetase
HCR	haem controlled repressor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
HMG-CoA	β-hydroxy-β-methylglutaryl-CoA
hnRNA	heteronuclear RNA
lnRNA	low-molecular weight RNA
K_m	Michaelis-Menten constant
POPOP	1,4-bis-(2-(5-phenyloxoly)-benzene
PPO	2,5-diphenyloxazole
R.I.D.	radial immunodiffusion
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
T.I.	turnover index

INTRODUCTION

INTRODUCTION

Hepatic lipogenesis is a complex process controlled by a multitude of short-term (Geelen *et al.*, 1980) and long-term (Volpe and Vagelos, 1974) regulatory mechanisms. The short-term regulation involves modulation of enzyme activity by substrate supply, allosteric effectors and covalent modifications; while the long-term regulation involves fluctuations in the absolute amounts of the cellular enzymes. These fluctuations in enzyme quantities occur through variations in the levels of enzyme synthesis and degradation which are in turn controlled by a variety of cellular mechanisms. These controls on lipogenesis in general and fatty acid synthesis in particular are irrevocably interrelated with the processes controlling the other aspects of cellular metabolism.

Fatty acid synthetase (FAS) is considered to be a primary site of long-term regulatory controls in hepatic fatty acid synthesis. Previous studies have shown that refeeding a fat-free diet following a period of starvation resulted in a dramatic increase in liver fatty acid synthetase (Hicks *et al.*, 1965; Burton *et al.*, 1969); this phenomenon is known as adaptive synthesis. The rapid accummulation of hepatic FAS during adaptive synthesis results from an enhanced rate of synthesis on non-membrane bound ribosomes (Yu and Burton, 1974). Degradative influences have also been observed in a number of systems showing variations in FAS levels (see Volpe and Vagelos, 1974; Wilde *et al.*, 1980).

The systemic response to inflammation, induced by a subcutaneous injection of oil of turpentine, is accompanied by an increase in the synthesis of a number of secretory proteins, termed acute phase

reactants, which are synthesized on membrane-bound polyribosomes. Among these acute-phase proteins is α_1-acid glycoprotein which increases 5-fold by 12 h following the induction of experimental inflammation (Jamieson and Ashton, 1973; Jamieson, 1977).

Shutler *et al.* (1977) showed that the increase in hepatic FAS characteristic of adaptive synthesis is suppressed by experimental inflammation to an extent which depends on the time interval between the onset of fat-free feeding and the induction of inflammation. Similarly the acute-phase response of α_1-acid glycoprotein was suppressed by adaptive synthesis to an extent dependent upon the same time interval but in an opposite fashion. When inflammation was an early event after refeeding, adaptive synthesis of FAS was suppressed but α_1-acid glycoprotein and serum albumin (liver-synthesized protein which is not an acute-phase reactant) responded in a manner characteristic of inflammation alone. The reverse was true when inflammation was a late event after refeeding. Shutler *et al.* (1977) also found an increase in the proportion of non-membrane bound polysomes as compared to membrane-bound polyribosomes with adaptive synthesis. The suppression of the adaptive synthesis of FAS was accompanied by an apparent increase in the membrane-bound polysome fraction. These results suggest that the mechanism of suppression involves the cellular protein-synthesizing machinery. More recently (Lombart *et al.*, 1980) an increase in the components of the biosynthetic pathway for glycoproteins has been observed with inflammation. These variations in the levels of the protein-synthesizing apparatus for the two types of proteins is probably more of a longer-term stabilization (or an effect) of long-term changes rather than an immediate long-term effector.

It appears likely that the interactions of the two processes, adaptive synthesis and the acute-phase response, occur at the level of the processes controlling the turnover of the two different proteins. This thesis is an attempt to further delineate the mechanisms of the interactions between the two processes. The variations in enzyme levels, changes in relative rates of enzyme synthesis and degradation, and variations in the levels of translatable messenger RNA were investigated.

Also, because adaptive synthesis and the acute-phase response are apparently under hormonal control (Volpe and Marasa, 1975; John and Miller, 1969), the changes in liver or serum levels of cortisol, insulin, glycogen, glucose and cAMP were investigated. A preliminary study on some other aspects of lipogenesis was also performed. This included studying variations in total lipid, cholesterol and triglyceride levels and the changes in ATP-citrate lyase and acetyl-CoA carboxylase levels.

HISTORICAL

HISTORICAL

Fatty acids are commonly found in all types of cells, usually bound in an ester linkage with other components to form the various classes of cellular lipids. These lipids are essential components of all living cells, functioning as an energy store and playing an important role in biological membranes. The biosynthesis of fatty acids, and lipogenesis in general, has a remarkable similarity in most organisms studied. The halobacteria are an exception to this rule, synthesizing polyisoprenoid carbon chains instead of fatty acids under normal growth conditions (Oesterhelt, 1976).

These fatty acids, in the form of triacylglycerols, represent a concentrated form of cellular energy which on a by-weight basis are a much greater source of energy than either carbohydrates or proteins (Stumpf and Barber, 1960). An important factor in the survival of a living organism is its ability to regulate this energy storage and control its utilization in times of food shortage.

I. FATTY ACID SYNTHETASE: STRUCTURE AND MECHANISM

The process of the mobilization of the energy stored in fats occurs via the β-oxidation pathway. The essential features of this pathway were first formulated in 1904 by Knoop and were not fully confirmed for fifty years (Green, 1954; Lynen, 1954). The original mechanism proposed for fatty acid biosynthesis was a reversal of the β-oxidation pathway but a number of early observations were inconsistent with this view. Gibson *et al.* (1958) observed that fatty acid synthesis in partially purified extracts of avian liver required ATP and bicarbonate, two components not accounted for by the β-oxidation scheme. This

led to the discovery of a biotin-containing enzyme called acetyl-CoA carboxylase which functions in the carboxylation of acetyl-CoA to malonyl-CoA (Wakil and Gibson, 1960). Acetyl-CoA carboxylase is now recognized as the catalyst of the first enzymatic step in the two step process of fatty acid synthesis. The malonyl-CoA acts as the two-carbon donor for chain elongation resulting in the production of long-chain fatty acids. The CO_2 fixed in the first reaction is ejected and is important from a thermodynamic point of view. This is the second step in fatty acid biosynthesis and is catalyzed by a second enzyme (or enzyme group) known as fatty acid synthetase or FAS (Lynen, 1961; Wakil, 1961; Vagelos, 1964). The reaction requires malonyl-CoA as the two-carbon donor, NADPH as the source of reducing power, and a short chain acyl-CoA acting as a priming substrate. The stoichiometry was first elucidated with enzyme systems from pigeon (Wakil, 1961) and rat liver (Brady *et al.*, 1960):

Acetyl-CoA Carboxylase

$$ATP + HCO_3^- + \text{Biotin-Enzyme} \rightleftharpoons CO_2\text{-Biotin-Enzyme} + ADP + Pi$$

$$CO_2\text{-Biotin-Enzyme} + CH_3COSCoA \rightleftharpoons \text{Biotin-Enzyme} + COOHCH_2COSCoA$$

Fatty Acid Synthetase

$$CH_3COSCoA + 7\ COOHCH_2COSCoA + 14\ NADPH + 14\ H^+$$

$$\longrightarrow CH_3CH_2(CH_2CH_2)_6CH_2COOH + 8\ CoASH + 14\ NADP^+ + 7CO_2 + 6H_2O$$

The fatty acid synthetase system has been isolated from a variety of plants, animals, insects and microorganisms (see Tables I and II for references) and found to exist in at least two distinct forms.

The enzyme system of *E. coli* (Alberts *et al.*, 1963, 1964) consists of seven individual protein components. One of these components is a low molecular weight acyl carrier protein (ACP) (Majerus *et al.*, 1965)

while the other six make up the different catalytic activities necessary to produce a long-chain fatty acid. Majerus *et al.* (1965) demonstrated that the intermediates in fatty acid biosynthesis exist as acyl groups bound in thioester linkage to the prothetic group, 4'-phosphopantetheine, of the acyl carrier protein. The ACP-intermediates play a central role in the reaction sequence which is shown below (Majerus *et al.*, 1964; Majerus and Vagelos, 1967; Wakil *et al.*, 1967):

$$CH_3CO\text{-}S\text{-}CoA + HS\text{-}ACP \xleftarrow{\text{Acetyl-CoA-ACP transacylase}} CH_3CO\text{-}S\text{-}ACP + CoA\text{-}SH$$

$$CH_3CO\text{-}S\text{-}ACP + HS\text{-}E_{cond} \rightleftharpoons CH_3CO\text{-}S\text{-}E_{cond} + ACP\text{-}SH$$

$$HO\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2CO\text{-}S\text{-}CoA + HS\text{-}ACP \xleftarrow{\text{Malonyl-CoA-ACP transacylase}} HO\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2CO\text{-}S\text{-}ACP + CoA\text{-}SH$$

$$HO\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2CO\text{-}S\text{-}ACP + CH_3CO\text{-}S\text{-}E_{cond} \rightleftharpoons CH_2COCH_2CO\text{-}S\text{-}ACP + HS\text{-}E_{cond} + CO_2$$

$$CH_3COCH_2CO\text{-}S\text{-}ACP + NADPH + H^+ \xleftarrow{\beta\text{-Ketoacyl-ACP reductase}} CH_3CHOHCH_2CO\text{-}S\text{-}ACP + NADP^+$$

$$CH_3CHOHCH_2\text{-}CO\text{-}S\text{-}ACP \xleftarrow{\beta\text{-Hydroxyacyl-ACP dehydrase}} CH_3CH = CHCO\text{-}S\text{-}ACP + H_2O$$

$$CH_3CH = CHCO\text{-}S\text{-}ACP + NADPH + H^+ \xleftarrow{\text{Enoyl-ACP reductase}} CH_3CH_2CH_2CO\text{-}S\text{-}ACP + NADP^+$$

$$\text{Fatty acyl-S-ACP} + H_2O \xleftarrow{\text{Fatty acyl-S-ACP thioesterase}} \text{Fatty acid} + ACP\text{-}SH$$

The reaction sequence is initiated by the transfer of an acetyl-group from thioester linkage with CoA to thioester linkage with ACP, by the enzyme acetyl-CoA ACP transacylase. The acetyl group is

Table 1. Type II Fatty Acid Synthetases - Individual Enzymes

Source	Reference
Escherichia coli *Clostridium sp.* *Pseudomonas sp.* *Bacillus subtilis*	Lennarz *et al.* (1962) Goldman *et al.* (1963) Pugh *et al.* (1966)
Avocado mesocarp	Overath and Stumpf (1964)
Lettuce chloroplasts	Brooks and Stumpf (1966)
Spinach chloroplasts	Simoni *et al.* (1967)
Euglena gracilis (photoauxotrophic)	Delo *et al.* (1971)
Chlamydomonas reinhardi	Sirevåg and Levine (1972)

transferred to a -SH group of β-ketoacyl ACP synthase (condensing enzyme), and ACP is liberated to accept a malonyl group transferred from malonyl-CoA ACP transacylase. The condensing enzyme produces the β-ketoacyl derivative by the condensation of the acetyl-enzyme and malonyl-ACP with the elimination of CO_2. This is followed by a reduction, a dehydration and a second reduction forming a saturated acyl-ACP thioester which is again transferred to the condensing enzyme and the ACP released. The ACP accepts another malonyl group and the process is repeated until a C_{16} or C_{18} fatty-acyl ACP is formed. This is hydrolyzed by a thioesterase to yield a free fatty acid although in the *in vivo* situation it may be transferred directly into phospholipid biosynthesis (Ailhaud and Vagelos, 1966).

The enzymes of most bacteria and plants exist as monofunctional and separable protein units. Table I lists the different organisms from which this type of FAS system has been isolated (termed type II based on their behavior *in vitro* as described by Brindley *et al.*, 1969). In contrast to these monofunctional protein units; most animals, eukaryotic microorganisms and some bacteria have FAS complexes which operate as a multifunctional unit. This group termed type I is listed in Table 2.

Acyl carrier proteins from the bacterial and plant systems exist as monofunctional units which are similar in amino acid composition and primary structure (for review see Prescott and Vagelos, 1972). The ACPs from different sources function interchangeably but the products formed vary according to the ACP used (Simoni *et al.*, 1967).

Table 2. Type I: Multienzyme complexes

Source	Molecular Wt. Native FAS	Molecular Wt. Subunit	Reference
Yeast	2.3×10^6	1.8×10^5, 1.85×10^5	Lynen, 1961; Schweizer, 1973
Rat Liver	5.4×10^5	2.5×10^5	Burton *et al.*,1968; Stoops *et al.*,1975
Rabbit Liver	4.5×10^5	2.25×10^5	Demassieux and Lachance 1974
Pigeon Liver	4.5×10^5	2.2×10^5	Hsu *et al.* 1965; Butterworth *et al.* 1967
Human Liver	4.1×10^5	2.3×10^5	Roncari 1974; Qureshi *et al.* 1976
Chicken Liver	5.1×10^5	2.5×10^5	Hsu & Yun, 1970
Rat Mammary Gland	4.8×10^5	2.4×10^5	Smith & Abraham, 1970
Rabbit Mammary Gland	4.8×10^5	2.4×10^5	Grunnet & Knudsen, 1978
Bovine Mammary Gland	5.3×10^5	2.4×10^5	Maitra & Kumar 1974
Goat Mammary Gland	4.8×10^5	2.3×10^5	Grunnet & Knudsen, 1978
Mouse Brain	5.0×10^5		Bloch & Vance 1977
Goose Uropygial Gland	5.5×10^5	2.7×10^5	Buchner & Kolattukudy,1976
Ceratitis capitata	5.6×10^5	2.5×10^5	Bloch & Vance 1977
Mycobacterium smegmatis	1.4×10^6	2×10^5	Vance *et al.* 1973
Corynebacterium diphtheriae	2.5×10^6		Knoche & Koths 1973
Penicillium patulum	2.6×10^6		Dimroth *et al.*1972
Pythium debaryanum	$\sim 4.0 \times 10^6$		Law & Burton 1972
Euglena gracilis	1.7×10^6		Delo *et al.* 1971
Rat erythrocyte	3.1×10^5	1.25×10^5	Jenik & Porter 1979

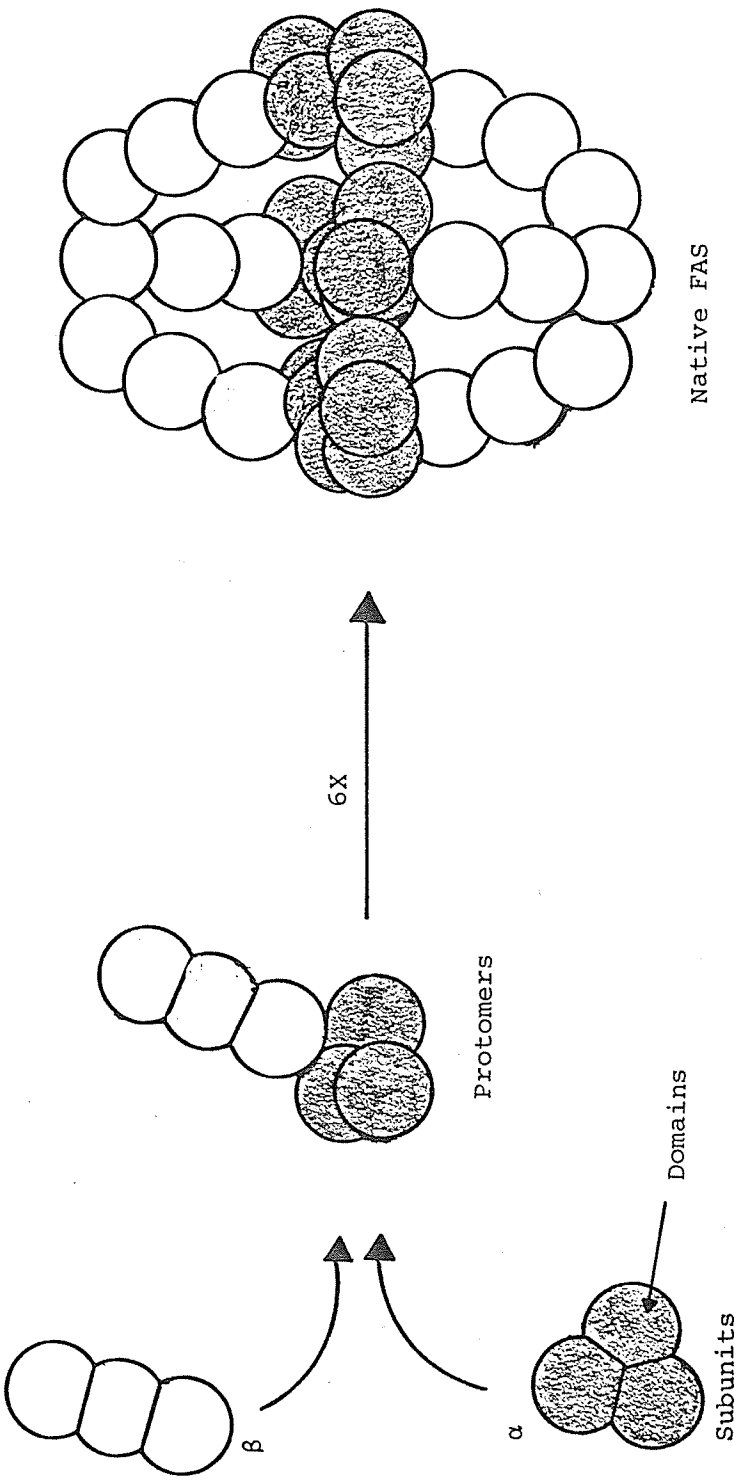
1.1 Multifunctional FAS Complexes

The yeast FAS has been crystallized and analyzed by electron-microscopy, small angle X-ray analysis (Lynen, 1967), immune-electron microscopy (Lynen, 1980) and by some elegant biochemical means (Engeser *et al.*, 1977; Weiland *et al.*, 1979). The current view of the yeast enzyme (Lynen, 1980; Schweizer, 1980) is that it is composed of two non-identical subunits (α and β) which are coded for by two unlinked genes. Structurally the complex consists of 6 protomers, each composed of an α and a β subunit, arranged as illustrated in Figure 1: (Weiland *et al.*, 1978; Stoops and Wakil, 1980). Other studies have separated the different enzymatic activities onto the different subunits and identified domains or globular regions which appear to contain the individual activities (Lynen, 1980). Between these domains appear regions which are preferentially susceptible to protease attack explaining the earlier identification of 7 different N-terminal amino acids from the yeast enzyme (Lynen, 1967).

Other multifunctional FAS complexes have been purified to homogeneity (see Table 2 for references) with the most extensive work done on the pigeon liver enzyme (for reviews see Volpe and Vagelos, 1976; Katiyar and Porter, 1977). In most cases, FAS can be isolated as a single functional unit with a molecular weight of approximately 500,000, which behaves as a single protein on ion exchange chromatography, gel filtration and centrifugation. This molecule is competent in the catalysis of fatty acid synthesis from acetyl- and malonyl-Coa and NADPH. Some early evidence indicated the FAS structure was composed of a tightly assembled complex of individual proteins which could be visualized by gel electrophoresis (Yang *et al.*, 1966; Bratcher and Hsu,

Figure 1: Schematic representation of a model of the Yeast fatty acid synthetase complex (from Lynen, 1980).

The proposed three-dimensional model has the complex built from 6 protomers, each protomer consisting of one α and one β subunit (each consisting of a number of domains) which are connected in the form of the letter V. The native FAS molecule consists of a hexagonal basic disc (of α subunits) from which 3 β subunits protrude upwards and 3 β subunits protrude downward. The individual subunits consist of regions or domains, containing the enzymatic activities, which are joined to each other by regions which are particularily sensitive to proteolytic attack.


Native FAS
6x
Protomers
Domains
Subunits
β
α

1975). Consistent with the above proposal are reports of the experimental isolation of ACP from yeast (Willecke *et al.*, 1969) and rat, pigeon, chicken and human liver FAS (Qureshi *et al.*, 1976). More recent experimental evidence, however, is inconsistent with the above view and the current hypothesis is that the enzyme is composed of two polypeptides, each having a molecular weight one-half that of the enzymatically active complex (see Wakil, 1970; Bloch and Vance, 1977; Hardie and Cohen, 1978).

Early work indicated liver FAS was easily dissociated into apparently inseparable one-half molecular weight subunits (Hsu *et al.*, 1965; Burton *et al.*, 1968). The dissociated preparation is capable of all the partial reactions of fatty acid synthesis except for the β-ketoacyl thioester synthase activity, and therefore lacks overall FAS activity.

As previously mentioned, genetic evidence with yeast indicates the existence of two different subunits with the ACP existing as a domain of one or both subunits (Schweizer *et al.*, 1973; Schweizer, 1980). Some experiments implied a similar arrangement in other multifunctional FAS complexes. Qureshi *et al.* (1976b) reported separating two distinct subunits from pigeon liver FAS by affinity chromatography and identified one mole of 4'-phosphopantetheine per mole of FAS complex (500,000 Mw species) (see Katiyar and Porter, 1977). This work remains unconfirmed by others.

More recently, considerable evidence has accumulated indicating that the half-molecular weight species are single polypeptides which are probably identical. Thus, careful analysis of enzyme from various sources indicates the presence of one mole of 4'-phosphopantetheine per mole of subunit, i.e. two per intact, active FAS complex (Buchner and

Kolattukudy, 1976; Stoops *et al.*, 1978; Wood *et al.*, 1978).

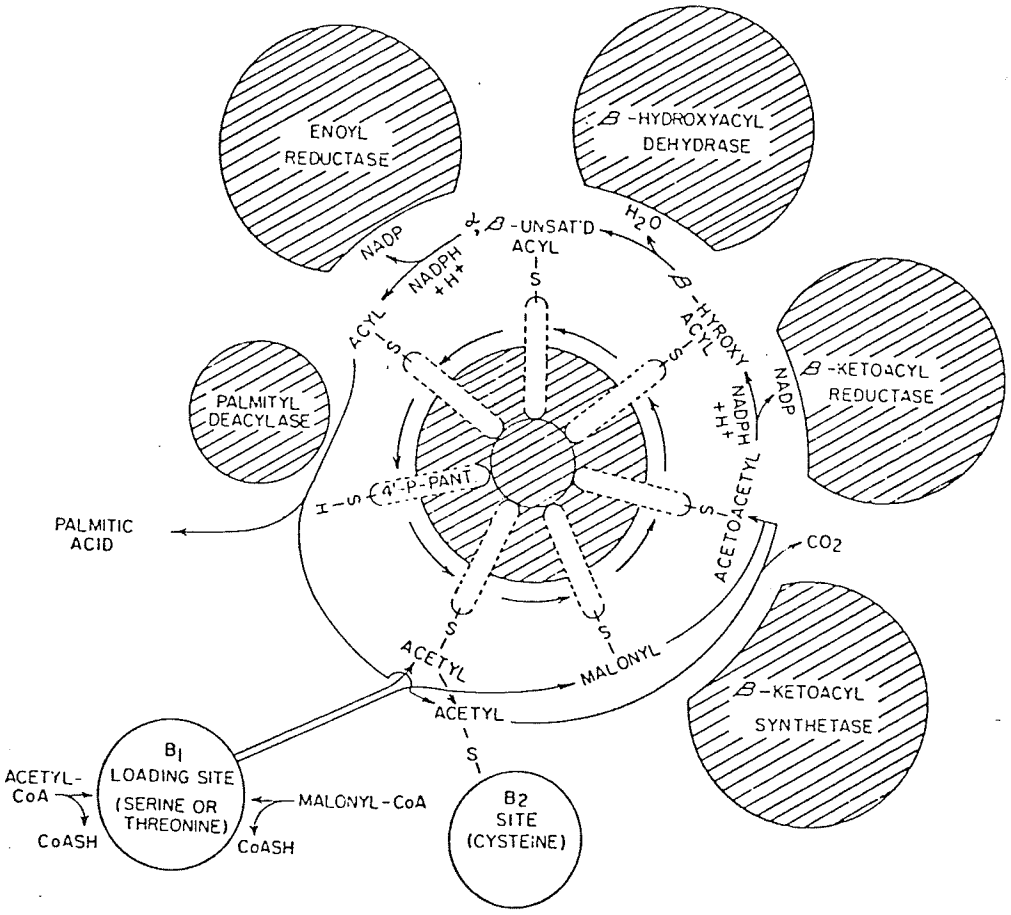
Studies of limited proteolysis with elastase (Guy *et al.*, 1978) and trypsin (Smith and Stern, 1979) indicate the two subunits are susceptible to attack at identical locations. Circular dichroism studies of FAS from an insect (Gavilanes *et al.*, 1978), and the requirement for two moles of chloroacetyl-CoA (Kumar *et al.*, 1980) or dibromoacetone (Wakil and Stoops, 1980) per mole of FAS for complete enzyme inactivation lend further support to the homodimer hypothesis. NADPH binding studies (Dugan and Porter, 1970; Yu and Burton, 1972) identified two pairs of binding sites per mole of enzyme, while more recent studies using pyridoxal phosphate to block the enoyl reductase binding site have identified two identical binding sites per subunit (Poulose *et al.*, 1980).

The reaction mechanism for pigeon liver FAS is essentially the same as the yeast and *E. coli* enzyme. A number of sequential reactions occur in which the intermediates are bound by thioester linkage to 4'-phosphopantetheine. The reaction is initiated by the transfer of acetyl- and malonyl-groups to the enzyme via a serine "loading site" with the subsequent transfer by the ACP to an "acyl storage site" on the condensing enzyme (see Figure 2 from Porter *et al.*, 1971). This is followed by a cycle of reduction, dehydration, a second reduction and condensation until free palmitic acid is released. Recently a CoA requirement for the final thioesterase reaction has been demonstrated (Lin and Srere, 1980; Lin *et al.*, 1980).

Recent studies indicate the active form of the FAS molecule of animal tissue is a dimer of two identical multifunctional polypeptides with each subunit containing the same seven catalytic centers and a

Figure 2: A proposed mechanism of fatty acid synthesis (adapted from Katiyar and Porter, 1977).

The individual reactions of the fatty acid synthetase complex are depicted as occurring in a cyclic fashion resulting in the formation of palmitic acid from malonyl-CoA, acetyl-CoA and NADPH. The reductions and dehydrations are depicted as occurring on a rotating (or freely moving) 4'-phosphopantetheine prosthetic group.


ENOYL
REDUCTASE
β-HYDROXYACYL
DEHYDRASE
α,β-UNSAT'D
ACYL
NADP
NADPH
+H+
H2O
ACYL
β-HYROXY
ACYL
PALMITYL
DEACYLASE
β-KETOACYL
REDUCTASE
NADP
NADPH
+H+
4'-P-PANT
PALMITIC
ACID
ACETOACETYL
CO2
ACETYL
MALONYL
ACETYL
β-KETOACYL
SYNTHETASE
B1
LOADING SITE
(SERINE OR
THREONINE)
ACETYL-
CoA
CoASH
MALONYL-CoA
CoASH
B2
SITE
(CYSTEINE)

prosthetic group (see preceding discussion and figure 2). Evidence indicates the dissociated monomers are inactive in fatty acid synthesis suggesting a functional interaction between the two polypeptides. The two prosthetic groups may be involved in initiation or the dimer may create a catalytic center necessary for activity (Stoops *et al.*, 1979). Wakil and Stoops (1980) have proposed a dimeric form in which the β-ketoacyl synthetase site of one subunit is positioned close to the 4'-phosphopantetheine of the other subunit. Chain elongation occurs by the transfer of the acyl group from one subunit to the other during each round of elongation. Another model proposes the substrates bind to one of the subunits to initiate synthesis while the second subunit acts as a coordinator in the reaction cycle (Srinivasan and Kumar, 1981).

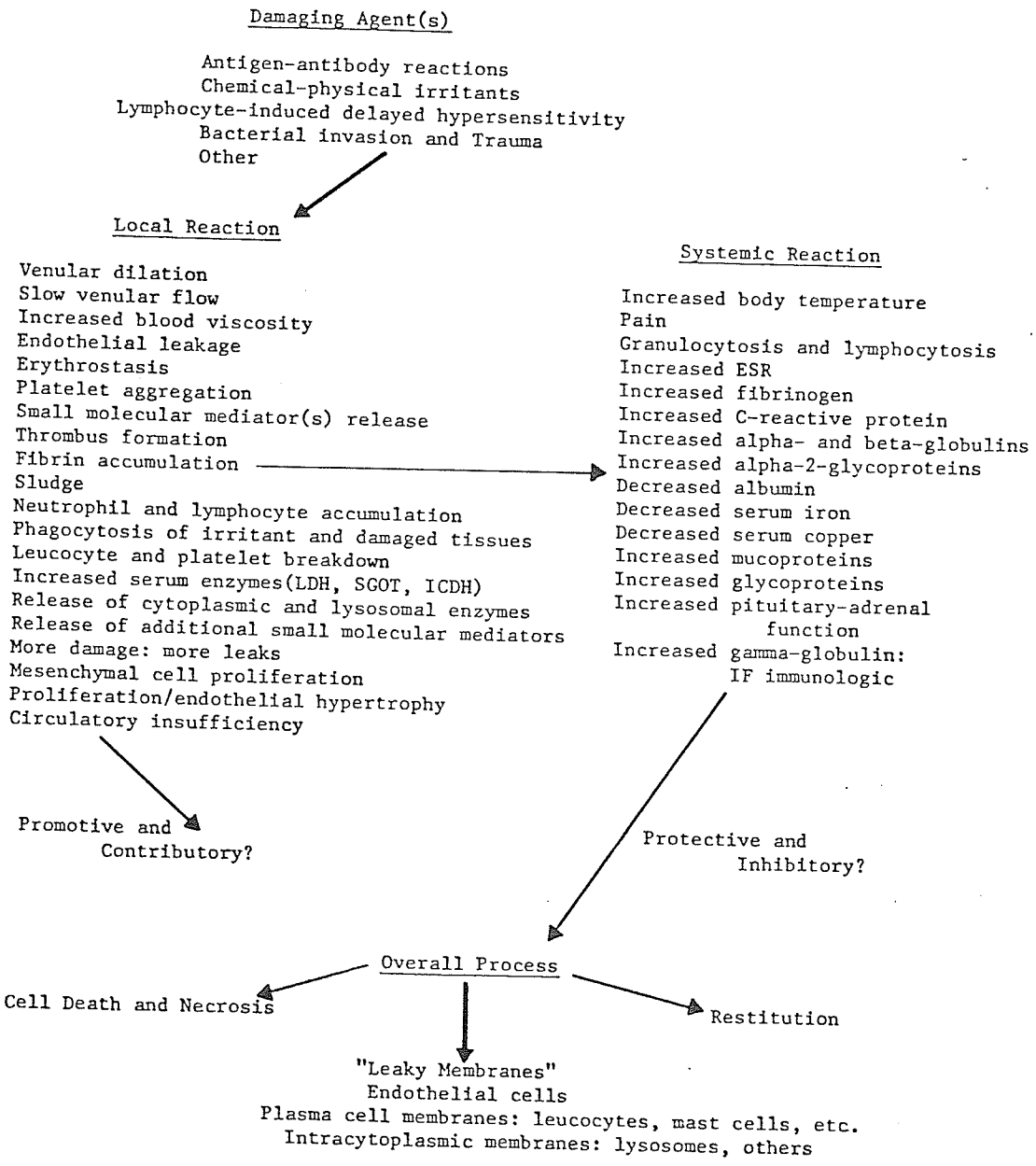
The structural and mechanistic complexity of the enzyme is readily evident from the preceding discussion. The hypothesis that the multifunctional enzyme is an evolutionary development is intuitively attractive although the significance of the homodimer in birds and mammals and the large yeast molecule is unknown. The process of gene duplication and gene rearrangement during development may provide some insights into the variations observed in the FAS enzymes and genes (Kirschner and Bisswanger, 1976).

2. GLYCOPROTEINS AND THE RESPONSE TO INFLAMMATION

All serum and plasma proteins, with the exception of albumin and some lipoproteins, appear to be glycosylated although the function of this glycosylation is not well understood (Geison and Gordon, 1978). Structurally and functionally glycoproteins are a diverse group which exhibit great variations in carbohydrate type and content (for reviews see Struck and Lennarz, 1980; Kornfeld and Kornfeld, 1976, 1980).

Figure 3: Schematic representation of the Inflammatory Process, including the localized reaction and the acute systemic response (from Glenn *et al.*, 1968).

THE "INFLAMMATORY PROCESS"



The early studies of Miller and Bale (1954) indicated the liver was the principal site of biosynthesis for all the plasma proteins except the immunoglobulins. More recent studies have confirmed this and shown that the levels of the plasma proteins are closely regulated by the physical status of the animal (Koj, 1974; Jamieson and Ashton, 1973a,b; Moscarello *et al.*, 1967; Maung *et al.*, 1968). Changes in levels can be linked to challenges such as infection or tissue injury indicating protection and repair may be primary functions of the plasma proteins. Trauma of any sort, such as inflammation, rapidly increases the levels of a number of plasma proteins, called the acute phase reactants, which function in a complex series of interrelated events in defense and repair.

The inflammatory response may be induced by a wide variety of conditions including chemical agents, neoplastic diseases, rheumatoid arthritis, etc. (Gordon, 1970; Tomasi, Jr., 1977). This response can be divided into two reactions (see Figure 3); a localized reaction at the immediate site of tissue damage and the systemic response which is induced by the release of components from the local reaction (Glen *et al.*, 1968). The local reaction may manifest itself by forming a tumor-like mass of collagen and leukocytes, called a granuloma, at the site of infection or trauma (Warren, 1972). The systemic response to the localized reaction may be acute or it may develop into a chronic response (Houck, 1967). The acute response appears to involve an interrelated series of events (for review see Beisel, 1975). One series of events follows a characteristic pattern independent of the type of inflammatory trauma, while another involves specific responses dependent upon the type or degree of inflammation (Galin *et al.*, 1969).

The hepatic reaction to inflammation depends upon the release of a

number of mediators from the site of the localized reaction. The release of these factors is attributable to destructive events such as permeabilization of cellular membranes, phagocytosis, vasodilation, increased vascular permeability, etc.; and involves histamine, serotonin, elements of the kinin system and other factors as illustrated in Figure 4. These mediators including endogenous pyrogen (EP) and leukocyte endogenous mediator (LEM) act on distant organs or cells to induce the acute response (Figure 4) (Beisel, 1975). Prostaglandins (Vance, 1972) and neutrophil-generated superoxide (Petrone, 1980) have also been implicated as mediators in the inflammatory response, together with a variety of other hormonal effectors.

2.1 Hormonal Involvement in the Inflammatory Response

Insulin and glucagon have been implicated in the acute response although the involvement may be mainly due to their control over hepatic carbohydrate metabolism. In the face of an acute infection, liver metabolism faces extreme energy demands (i.e. to produce and secrete the acute phase reactants) which it copes with by resetting the hepatic processes to favor glycogenolysis (Tsung and Parersky, 1968), gluconeogenesis (Felig, 1973) and lipolysis (see review by Geelen, 1980). One response is an increased flux of amino acids from the plasma into the liver which seems to involve a glucagon-stimulated adenylate cyclase on hepatic cell membranes (Beisel, 1975).

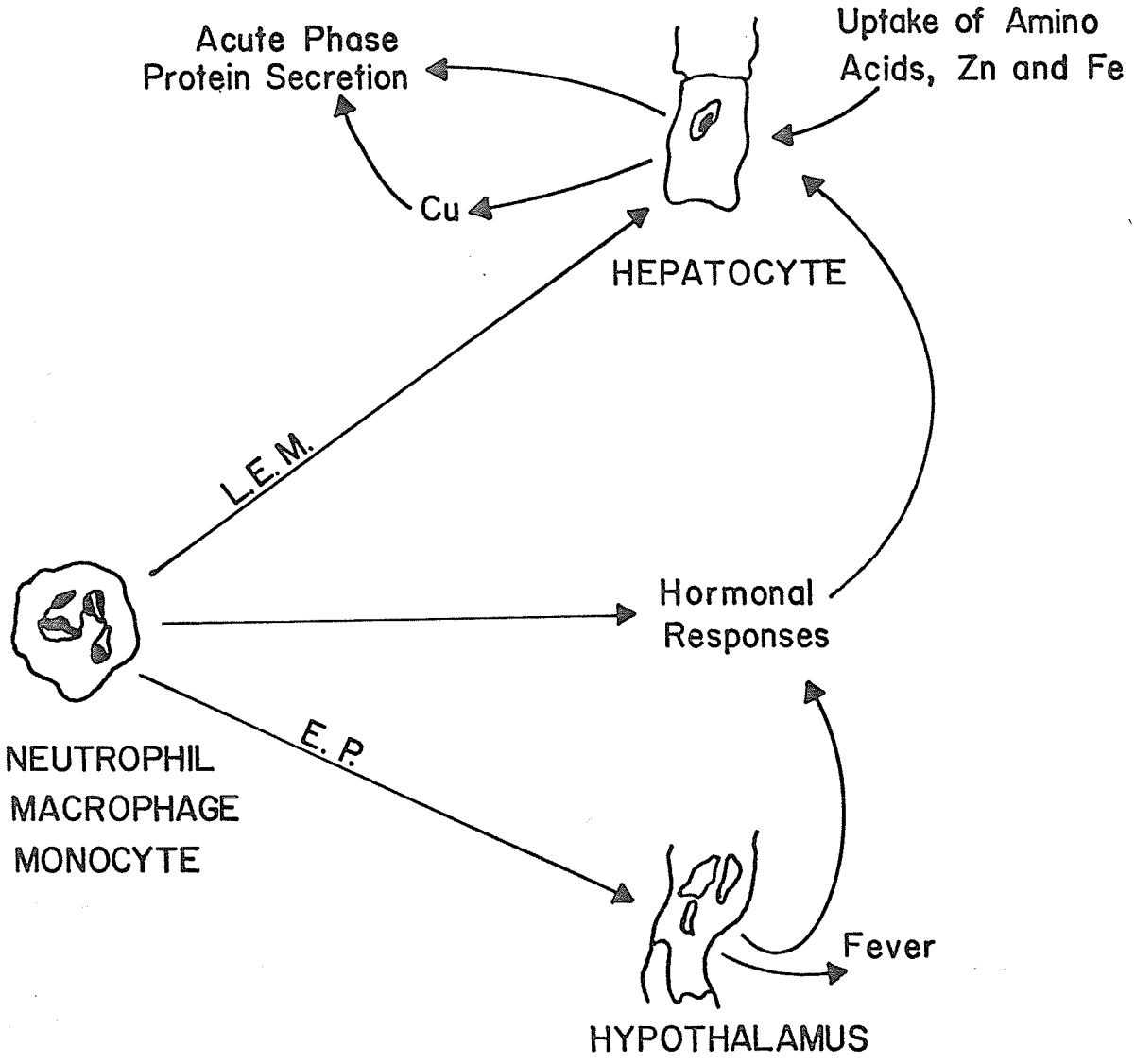
Insulin has been found to increase the rate of synthesis of total hepatic protein in general and not the acute phase reactants in particular (John and Miller, 1969). The same authors presented evidence that the effect may be partially due to a promotion of a positive nitrogen balance in the liver by insulin (John and Miller, 1974). Recent studies

Figure 4: Role of endogenous mediators in inducing the Acute response to inflammation (adapted from Beisel, 1975).

In addition to their phagocytic capabilities, certain cells act as fixed or mobile sentinels with the capability of releasing hormone-like mediators as signals to initiate generalized host responses in distant tissues.

E.P. Endogenous Pyrogen

L.E.M. Leukocyte Endogenous Mediator


Acute Phase
Protein Secretion
Uptake of Amino
Acids, Zn and Fe
Cu
HEPATOCYTE
L.E.M.
Hormonal
Responses
E.P.
NEUTROPHIL
MACROPHAGE
MONOCYTE
Fever
HYPOTHALAMUS

indicate diabetic patients have an increased susceptibility to infection through an impairment in the inflammatory response (Bagdade and Walters, 1980) which can be partially controlled by insulin treatment (Beisel, 1975). Elevated insulin levels have also been observed with bacterial infections (Kaminski *et al.*, 1979). MacGregor and co-workers (1978) found granulocyte adherence inhibited by cAMP and epinephrine, a hormone that increases cAMP levels. The action of insulin in granulocytes may be to lower cAMP levels and restore normal function. Another proposal has increased cAMP levels with inflammation functioning via a feedback mechanism to inhibit mediator release during an allergic reaction (Ortez, 1978).

Kaminski *et al.* (1979) also found a complicated relationship between starvation and inflammation. Under normal circumstances fasting causes a rise in the plasma levels of circulating ketone bodies and free fatty acids. Severe inflammation reduced the level of this effect and increased the levels of insulin and glucagon.

Studies with glucocorticoids have also given variable results. Cortisol levels are increased during inflammation and it appears to be required for the maximal induction of the acute phase proteins *in vitro* and at least α_2-globulin *in vivo* (Weimer and Benjamin, 1966; Heim and Ellenson, 1967). Adrenalectomy of rats does not seem to diminish the increase in α_1-acid glycoprotein with inflammation (Peters *et al.*, 1971), while some studies have found below normal levels of cortisol during subacute or chronic inflammation (Beisel and Rapoport, 1969). Dannenberg (1979) has proposed glucocorticords function as anti-inflammatory agents.

Growth hormone alleviates the repression of albumin synthesis observed in hypophysectomized animals (John and Miller, 1974; Kernoff

et al., 1971) although under normal circumstances it appears to have no effect. In the case of the other plasma proteins, growth hormone may be required for maximal synthesis in normal and hypophysectomized rats. The thyroid hormones, triiodothyronine and thyroxine, respond in a characteristic pattern to naturally acquired infection (Lutz *et al.*, 1972). Thyroxine may be involved in hepatic synthesis of plasma proteins although the effect may involve insulin and cortisol (John and Miller, 1974). One proposal has the iodine derived from the thyroid hormones playing a bacteriocidal role in phagocytic cells (DeRubertis *et al.*, 1973).

Recent evidence also indicates the inflammatory response is impaired in tumor-bearing animals (Gorog and Kovac, 1979).

3. PROTEIN BIOSYNTHESIS: CONTROL AND MECHANISMS.

3.1 Glycoprotein Biosynthesis

Early reports indicated the increase in acute phase reactants was due to an increased synthesis of these plasma glycoproteins by the liver (Koj, 1974; Gordon, 1970). Increases have been observed for the glycoprotein fractions (Ashton *et al.*, 1970; Moscarello *et al.*, 1967), α_1-acid glycoprotein and α_2-macroglobulin (Jamieson *et al.*, 1970), haptoglobulin (Dagrelle *et al.*, 1969) and more recently major acute phase α_1-protein (Urban *et al.*, 1979). A recent report (Lombart *et al.*, 1980) indicates turpentine-induced inflammation causes an increase in some of the components involved in the biosynthetic pathway of hepatic glycoproteins. These reports suggest that a complex translational control mechanism operates during inflammation.

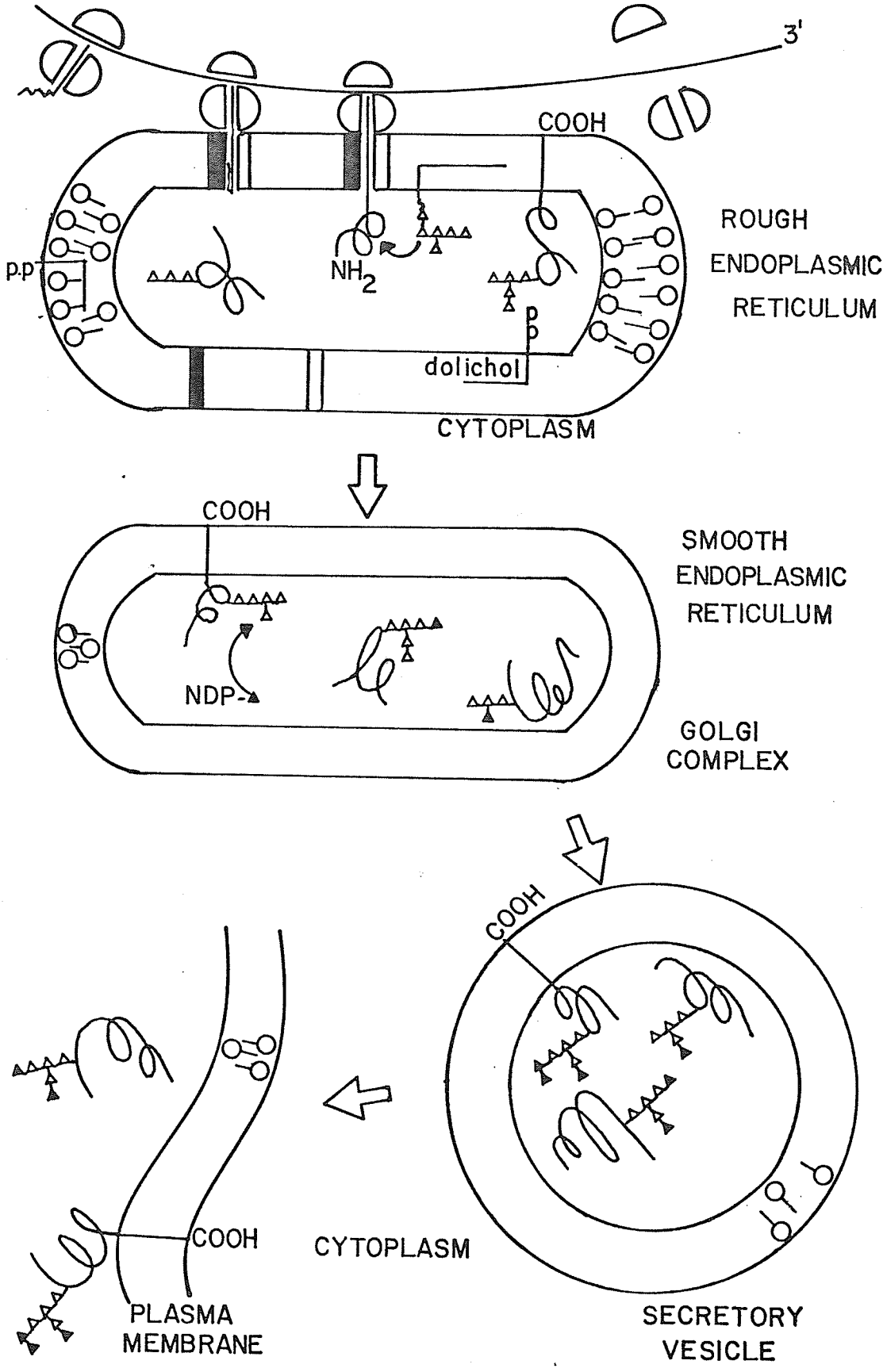
Experimental evidence points to a complex cellular organization existing in both prokaryotes and eukaryotes for synthesizing, processing and distributing proteins. The distribution of proteins to their sites

of utilization is thought to depend upon a topographical segregation of two populations of ribosomes, one existing free in the cytoplasm while the other is associated with various intracellular membranes particularly the endoplasmic reticulum (ER). In bacterial cells and reticulocytes, both of which lack an internal membrane system, a fraction of the ribosomes is found in association with the plasma membrane. Although functionally different, the two classes of ribosomes appear to possess similar morphological and biochemical characteristics (Venekateson and Steele, 1972; for review see Wool, 1979).

The concept that proteins destined for intramembrane or extracellular usage are synthesized on membrane-bound ribosomes developed from an observation of ribosome association with rough endoplasmic reticulum (Palade, 1955). Blobel and Sabatini (1971) proposed that the nascent peptide chains of secretory proteins contained some common feature which allowed them to interact with the membrane during their synthesis on membrane-bound ribosomes. Nilstein *et al.* in 1972 demonstrated that the cell-free translation product of light chain immunoglobulin messenger RNA was larger than the authentic isolated proteins. These authors and Blobel and Dobberstein (1975) postulated an N-terminal supplementary sequence that accounted for the larger size and served as a signal for membrane-association and protein secretion.

This "signal sequence" postulates the existence of a codon sequence located at the 5' end of the messenger RNA. Translation of this codon sequence yields a unique sequence of amino acids on the amino terminal end of the nascent peptide which functions in promoting the attachment of free ribosomes to specific receptors on the membrane. Ribosomal receptor proteins also associate with the membrane causing a "tunnel"

Figure 5: Model for the concerted translation and glycosylation of secretory and membrane glycoproteins (adapted from Struck and Lennarz, 1980).


3'
COOH
ROUGH
ENDOPLASMIC
RETICULUM
p-p
NH2
P
P
dolichol
CYTOPLASM
COOH
SMOOTH
ENDOPLASMIC
RETICULUM
NDP-
GOLGI
COMPLEX
COOH
COOH
CYTOPLASM
PLASMA
MEMBRANE
SECRETORY
VESICLE

formation through which the peptide chain passes. The signal peptide is proteolytically removed in the intracisternal space before translation and membrane transport is completed (see Figure 5) (Lingappa *et al.*, 1979b; Glabe *et. al.*, 1980).

A number of exceptions to the proteolytic processing of a signal peptide have been found. Ovalbumin is synthesized in its final and mature form and segregated into the reticulum membrane. Although its N-terminal sequence possesses no particular hydrophobicity, a characteristic of the signal sequence, an internal signal-peptide has been identified which is located more than 200 residues into the peptide chain (Steiner, 1979; Lingappa *et al.*, 1979). Other proteins have also been found to be translocated without the existence of a larger precursor form (see Waksman *et al.*, 1981). (For reviews see Blobel, 1979, 1980; Lennarz, 1979; Lewis, 1980; Struck and Lennarz, 1980).

Figure 5 illustrates a generalized mechanism of the biosynthesis and secretion of a typical glycoprotein (adapted from Struck and Lennarz, 1980). The processes of translation and glycosylation are found to be closely linked, with the addition of complex oligosaccharide units to the peptide chain occurring while translation is proceeding (Kiely *et al.*, 1976). Evidence with the vesicular stomatitis virus G protein indicates the oligosaccharide units are assembled on the cytoplasmic side of the rough endoplasmic reticulum while attached to a lipid carrier, dolichol phosphate. The dolichol-oligosaccharide is passed through the membrane and the oligosaccharide is transferred to an asparagine group on the emerging peptide (Li *et al.*, 1978; Kornfeld *et al.*, 1978; Tobas and Kornfeld, 1978). Processing of the oligosaccharide chain occurs in the rough E.R. and the glycoprotein moves into the smooth E.R.-Golgi complex where further processing occurs. The glycoprotein is then isolated inside a

secretory vesicle, in the case of secretory proteins, and in the vesicular membrane for membrane proteins (see Struck and Lennarz, 1980).

Other mechanisms exist with general or specific modifications of the above scheme. Jamieson (1977) has shown the initial glycosylation of rat α_1-acid glycoprotein occurs mainly or entirely after release of the nascent peptide from the ribosome site. Also, some kinetic observations imply there may be another transport pathway which bypasses the vesicular storage route (see Waksman *et al.*, 1981). Other discrepant results indicate that the biosynthesis of membrane glycoproteins may differ in their pathway of processing and insertion (see Morré *et al.*, 1979; Rothman and Fine, 1980).

Observation by electron microscopy indicates the extent of rough E.R. is dependent upon the function or functions the cell is fulfilling. Mammalian liver cells, which function both in secretion of plasma proteins and in intracellular processing of metabolites, have an extensively developed rough E.R. but not as extensive as cells specialized solely for secretion such as pancreatic exocrine or plasma cells (Palade, 1975). Cellular controls of the extent of segregation exist as antigenic stimulation of lymphocytes leads to the development of an extensive rough E.R. (see Lewis, 1980). The unstimulated lymphocytes contain a prominent Golgi apparatus but small amounts of rough E.R. In the case of hepatic cells and turpentine-induced inflammation, the amount of Golgi complex and smooth E.R., relative to rough E.R., increases perhaps implicating the existence of another regulatory mechanism (Turchen *et al.*, 1977). A more recent report (Lombart *et al.*, 1980) confirmed the increase in Golgi complex with inflammation and noted an increase in liver glycotransferase levels (originally observed by Turchen *et al.*, 1977).

Table 3(A). Secretory proteins synthesized by membrane-bound ribosomes.

Protein	Ratio of synthesis (bound/free)
Serum proteins	-
Albumin	7:1
	3:1
	5:1
	-
	5:1
	6:1 (or 1:1)
	6:1
	20:1
	30:1 (or 15:1)
	(98% mRNA in bound)
Immunoglobulin	-
	4:1
	Bound only
	Bound and free
	3:1
	-
Thyroglobulin	-
	8:1
	-
Casein	8:1
	20:1 (or 2:1)
α-Lactalbumin	-
α-Lactoglobulin	-
α-Fetoprotein	20:1
Fibroin	-
Prolactin	20:1
Procollagen	Bound only
Vitellogenin	-
Cellulase	4:1
Invertase	-
Alkaline phosphatase	∿4:1
Trypsinogen	Some synthesis by free
Chymotrypsinogen	Some synthesis by free

(B). Synthesis of Cytosol Proteins by Free and Membrane-Bound Ribosomes

Protein	Free	Bound	Free/bound
Ferritin	+		5:1
	+	(+)	1:5:1
	+	(+)	3:1
	+	(+)	4:1
	+	+	2:1
	+	(+)	3:1(or 1:1)
Albumin (not secreted)	+	-	5:1

...continued...

Table 3. (continued)...

Protein	Free	Bound	Free/bound
Myosin	+	-	-
Arginase	+	-	8:1 (mean)
Tubulin	+	+	1:1
Histones	+	+(loose only)	-
Ribosomal proteins	+	+(loose)	-
	+	-	-
Tyrosine aminotransferase	?	+	-
Serine dehydratase	+	+	-
Leghemoglobin	+	-	4:1

Note: (+) denotes low levels of synthesis detected.

Taken from Lewis (1980).

Table 3 summarizes the experimental results supporting a topographic segregation of proteins by translation on different groups of ribosomes.

The evidence that free ribosomes synthesize soluble intracellular proteins is less conclusive than for membrane-bound ribosomes and secretable proteins. Some soluble intracellular proteins are synthesized to some extent on membrane-bound ribosomes (eg. serine dehydratase, ferretin and histones) (see Lewis, 1980) although, as in the case of histone biosynthesis, the ribosomes are only loosely associated and membrane association may be an artifact of isolation (Zauderer *et al.*, 1973).

Some organelle proteins appear to be synthesized on both free and bound ribosomes. Catalase is found both in peroxisomes and free in the cytoplasm and is synthesized on both classes of ribosomes (Kashiwagi *et al.*, 1971). The majority of mitochondrial proteins are synthesized on bound ribosomes (Shore and Tata, 1977) but in some instances, eg. cytochrome *c*, both groups of ribosomes are utilized (Shore and Harris, 1977). A chloroplast protein, ribulobisphosphate carboxylase, is synthesized in a precursor form on free-ribosomes and transported to the chloroplast (Dobberstein *et al.*, 1977). A number of other cytoplasmic proteins (eg. catalase, *E. coli* alkaline phosphatase, tyrosinase) are found to exist in a precursor form and are proteolytically modified for activation (see Dean and Judah, 1980). An association between cytoplasmic ribosomes and the outer mitochondrial membrane of yeast has also been observed (Keyhani, 1973).

A basic premise of the signal hypothesis is that free and bound ribosomes exist in a dynamic equilibrium, with the signal for attachment residing in the messenger RNA and not in the protein-synthesizing

machinery. The kinetics of labeling of free and bound ribosomal RNA from normal rat livers are identical (Tanaka *et al.*, 1970) although under certain circumstances, eg. long-term protein deprivation and γ irradiation, small differences are observed (Ekren and Yatvin, 1972; Gaetani *et al.*, 1977). Studies on the distribution of ribosomes during the life-cycle of yeast supports the hypothesis of a precursor relationship between the two classes of ribosomes (Schneider *et al.*,, 1976). Contradictory results have appeared from comparisons of the protein complements of the two types of ribosomes (see Lewis, 1980). McConkey and Haber (1975) found membrane-bound ribosomes from Hela cells lacked the largest 60S protein while free ribosomes lacked a smaller protein. These results were not confirmed with rat liver cells (Lewis and Sabatini, 1977) which were found to have identical ribosomal proteins by one- and two-dimensional electrophoresis (Hoffman and Ilan, 1977). Ramsey and Steele (1977) have presented several differences between the 60S subunit proteins of free and bound ribosomes although these differences do not change the binding characteristics of the 60S subunit to the membrane (Suss *et al.*, 1966).

The possibility also exists that the cell contains two classes of bound ribosomes; one tightly bound and the other loosely bound. The loosely bound ribosomes are released by RNAse, EDTA and puromycin (Robash and Penman, 1971) and by high salt concentrations (Bleiberg *et al.*, 1972). One curious facet of these experiments is that different cells have a differing susceptibility to freeing these loosely bound ribosomes with these different agents (see Lewis, 1980).

3.2 Post-transcriptional and translational controls.

3.2.1. RNA Processing

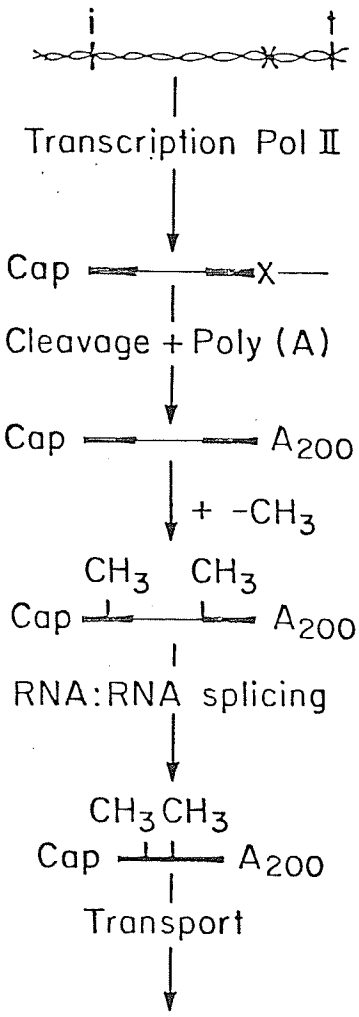
Prokaryotic controls of protein synthesis occur mainly at the transcriptional level while eukaryotic cells have both extensive and complex transcriptional and translational controls. Besides topographic segregation of ribosomes other controls exist intimately associated with the post-transcriptional and translational events.

Figure 6 illustrates the current view of the sequence of events occurring between transcription and the binding of a mature message to a ribosome (from Darnell, 1979). This process is believed to be common to all eukaryotic cells and involves transcription of a gene to yield a large primary transcription product or heteronuclear RNA (hnRNA) (see Darnell, 1978). The hnRNA is processed and modified in the nucleus and exported to the cytoplasm as a functional messenger RNA (mRNA). Controls exist in the efficiency of hnRNA processing, cytoplasmic distribution and stability of the message, and translational efficiency (Harpold *et al.*, 1981).

The nuclear processing of hnRNA involves at least four sequentially arranged steps: 1) addition of a 5' cap structure, 2) methylation of internal adenylate residues, 3) poly (A) addition to the 3' end and 4) cleavage and splicing reactions (see Revel and Groner, 1978; Nevins and Chen-kiang, 1981). The capping, internal methylation and polyadenylation appear to be involved in protection of the message from nuclease attack and in initiation of translation perhaps through ribosome affinity for the processed mRNA (see Pain and Clemens, 1980). Jeffery and Brawerman (1975) proposed that internal interactions with the poly(A) segment provided structural integrity for the message. The final step

Figure 6: Summary of mRNA biosynthesis based on work with Ad-2 transcription units and Chinese Hamster cells (from Darnell, 1979).

mRNA Biosynthesis



Initiation / Termination sites on DNA

2-20 kb, Avg 5 kb
Cap early
Abortion frequent

Within 1',
can be before termination

Methylation (m^6A) within 10-20'

5' to 25'

5' or less
Stochastic

in the processing of the nuclear message is the RNA cleavage: splicing reaction which forms a mature message from the high molecular weight precursor (for reviews see Georgiev and Ryskov, 1980; Sharp, 1981). Only a small portion of the transcriptional unit is conserved as a mature mRNA while the rest is removed and may be involved in regulatory processes. The splicing reaction has been observed with transfer-RNA precursors although the process appears different than for mRNA (Knapp *et al.*, 1978).

Splicing involves excision of an intervening sequence and rejoining the 5' and 3' ends of the adjacent exons. The possibility arises that the splicing reactions may occur on nuclear complexes, similar to cytoplasmic ribosomes, involving proteins and small RNA particles with catalytic and regulatory functions (Sharp, 1981).

3.2.2. Regulatory aspects of mRNA processing.

Mouse myeloma tumor cells differentiate from a cell-type producing a secretable immunoglobulin to a cell-type producing a membrane associated species. Early *et al.* (1980) found the RNA processing to be developmentally regulated, with one mRNA species formed by two additional splices of the hnRNA. Polyadenylation is suggested as determining the extent of splicing. Other shifts in the abundance of mRNAs, which have differential excision of intervening sequences, have been observed with adenovirus infection (Ziff, E.B., 1980). These results can be interpreted as either alterations in the rate of splicing or as variations in the messenger half-lives.

Figure 7 illustrates a number of proposed mechanisms involved in control of nuclear processing of hnRNA and cytoplasmic controls of mRNA translation (see Abelson, 1979; Naora and Deacon, 1981).

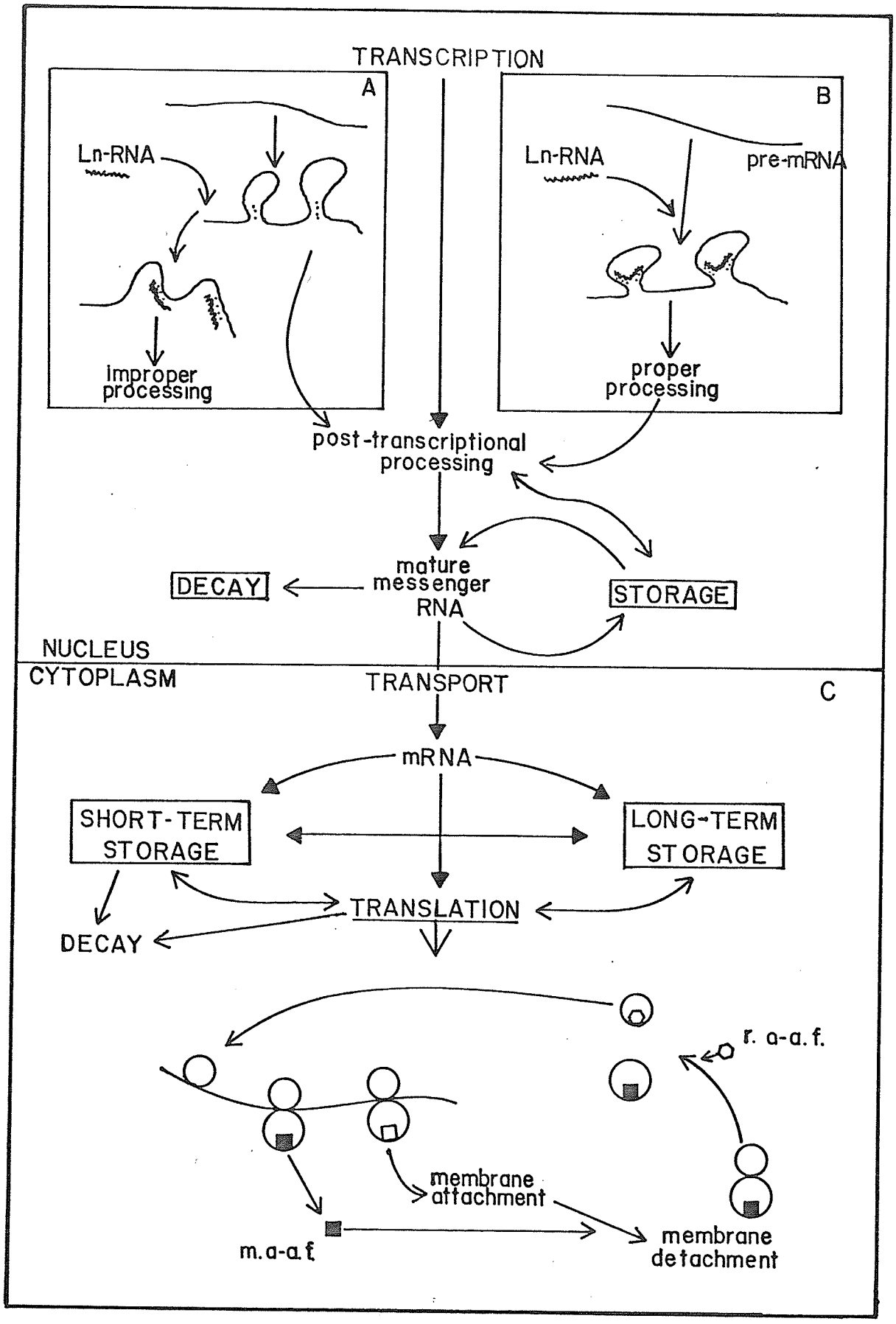
Figure 7: Schematic representation of some possible sites of translational, pre-translational (cytoplasmic) and post-transcriptional (nuclear) regulation.

Proposed regulation where Ln-RNA (low-molecular weight RNA) prevents splicing (Naora and Deacon, 1981) of heteronuclear RNA during processing (Figure 7A) or Ln-RNA enhances splicing (Lerner *et al.*, 1980) of heteronuclear RNA (Figure 7B).

Figure 7C from Lewis (1980) and Scherrer (1980).

m.a-a.f. membrane anti-association factor

r.a-a.f. ribosomal anti-association factor


TRANSCRIPTION
A
Ln-RNA
improper processing
B
Ln-RNA
pre-mRNA
proper processing
post-transcriptional processing
mature messenger RNA
DECAY
STORAGE
NUCLEUS
CYTOPLASM
TRANSPORT
C
mRNA
SHORT-TERM STORAGE
LONG-TERM STORAGE
TRANSLATION
DECAY
r. a-a. f.
membrane attachment
m. a-a. f.
membrane detachment

Low-molecular weight nuclear RNA (lnRNA) levels are subject to alteration by the physiological and pathological condition of the cell (Davidson and Britton, 1979). This observation has led to the two proposals, illustrated in Figure 7A and B, for the involvement of lnRNA in RNA splicing. One proposal has lnRNA binding in a regulatory function to prevent splicing (Naora and Deacon, 1981) while the second proposes an interaction of lnRNA with the mRNA excision site and an enhancement of the splicing reaction (Lerner *et al.*, 1980; Knowler and Wilkes, 1980).

Mechanisms appear to exist controlling storage of lnRNA in the nucleus and transport of the mRNA into the cytoplasm (see Scherrer, 1980). Differences in protein content of nuclear and cytoplasmic RNA, rapid turnover of mRNA proteins and modification of mRNA proteins by phosphorylation have been observed suggesting protein involvement in regulatory mechanisms (see Pain and Clemens, 1980). Hillar and Przyjemski (1979) predict control of gene expression by small peptides (termed deprimones), at both transcriptional and translational levels.

Figure 7(c) illustrates the proposed involvement of cytoplasmic factors in mRNA translation. Proteins (and possibly low-molecular weight RNA) may act in both short- and long-term storage and repression of a functional message in the cytoplasm (Rosenthal *et al.*, 1980; see Revel and Groner, 1978). Rosenthal *et al.* (1980) studying embryonic development of sea urchin eggs implicate increasing pH and decreasing intracellular calcium as controls in differential translation. Viral interference with host protein synthesis has been proposed to be due to an infection-induced sodium influx which selectively inhibits host mRNA translation (Carrasco, 1977).

3.2.3. Ribosomal and Transfer RNA Involvement.

Ogilive *et al.* (1979) has observed inhibition of the elongation step of translation with a moderate decrease in the aminoacylation of tRNA in mouse ascites tumor cells.

The synthesis of ribosomal RNA and protein appears to occur simultaneously and interference with the synthesis of one component adversely affects the synthesis of the other (see Pain and Clemens, 1980). Glucagon stimulation of cAMP dependent protein kinases is accompanied by phosphorylation of a 40S subunit protein and an increased synthesis of insulin, in hamster pancreatic islet cells (Jarrett and Penniston, 1978), leading to a suggestion of control by protein phosphorylation-dephosphorylation of ribosomal proteins (see Ochoa and deHara, 1979).

Two ribosomal anti-association factors are also illustrated in Figure 7c. A factor controlling disassociation of the ribosomal subunits allowing the smaller 40S subunit to bind another mRNA (Hunt, 1980) and a membrane antiassociation factor controlling release of the 60S subunit from the membrane (see Lewis, 1980) have been implicated in translational control.

Control of translation initiation in reticulocytes is modulated by a protein inhibitor, the haem controlled repressor (HCR), which is activated by haem deficiency. HCR possesses a cAMP independent protein kinase activity and acts primarily in phosphorylating and inactivating eIF-2, the initiation factor controlling met-tRNA binding to the 40S subunit (see Clemens, 1980). Walton and Gill (1975) have proposed eIF-2 control by the cellular energy charge through an increase in the GDP: GTP ratio.

3.3. Transcriptional controls.

Every eukaryotic cell contains a complete genome present in the zygotic nucleus but only a fraction is expressed in any cell or group of cells. The control of this expression, which is important in development, differentiation and varying responses to physiological stresses, depends upon differential expression of the genetic material (for reviews see O'Malley *et al.*, 1980; Butterworth and Beebee, 1980).

Variations in RNA polymerase activity under different conditions have been observed including amino acid starvation and refeeding (Grunnt *et al.*, 1976) and hormonal induction (Fuhrman and Gill, 1976).

Structurally DNA is present in two forms, one densely staining, heterochromatic and the other a diffuse fibrillar form with which RNA synthesis is associated (Littau *et al.*, 1964). This implies a physical segregation of functional DNA which occurs with development and differentiation and is subject to cellular controls. A study with chick oviduct and stimulation of ovalbumin production by progesterone suggests the steroid receptor protein functions as a helix destabilization agent which increases the gene's susceptibility to nuclease digestion and polymerase activity (Hughes *et al.*, 1981). Other work suggests a more complicated control exists. Variations in transcription rates for the 3 genes of the ovalbumin gene family are observed under different conditions (LeMeur *et al.*, 1981) and differences in RNA processing and stability of the gene products have also been predicted (McKnight and Palmiter, 1979).

Regulation by de-repressor RNA through a feed-back control mechanism has also been predicted (see Frenster, 1980).

Selective amplification of genes through duplication has been

recently observed for the dihydrofolate reductase (Alt *et al.*, 1978), aspartate transcarbamylase (Wahl *et al.*, 1979) and the metallothionien-I genes (Beach and Palmeter, 1981). This process may have developmental implications and has recently been implicated in the induction of carcinogenesis (Pall, 1981) but its importance in physiological responsiveness is not known.

3.4 Controls of Protein Degradation

Besides the mechanisms controlling protein synthesis examined in the previous sections, cellular protein degradation is now recognized as an important component in the long-term regulation of metabolism. It fulfills a number of functions including elimination of abnormal polypeptides and is involved in the cellular responsiveness to different stimuli (for reviews see Goldberg and St. John, 1976; Ballard, 1980; Millward, 1980).

A heterogeneity of degradation rates is observed with cellular proteins, with specific variations appearing within different fractions of the cell (Russell *et al.*, 1980). A number of generalizations for control of protein degradation have been proposed: 1) large proteins tend to be degraded more rapidly than smaller ones, 2) acidic proteins tend to be degraded more rapidly than neutral or basic ones (Dice *et al.*, 1979), 3) glycoproteins are degraded more rapidly than non-glycosylated proteins (Kalish *et al.*, 1979) and 4) proteins with a higher proportion of surface hydrophilic regions are degraded faster (Segal *et al.*, 1976). Dice *et al.* (1978) found that the general correlations between protein structure and degradation were different in the liver and muscle of diabetic or starved animals, but the same in the brain of the two types. Other studies with stress-enhanced

degradation in plant tissues have shown no variations in the correlations, as compared to non-stressed tissue (Cooke and Davies, 1980). Variations of the correlations in different cellular fractions have also been proposed (see Cooke and Davies, 1980).

The molecular basis of degradation is believed to involve two components, one the lysosomes containing a variety of hydrolytic enzymes and the other a specific non-lysosomal component (see Ballard, 1980). Variations in the degradation rates of specific proteins or groups of proteins may involve cofactor or metabolite stabilization or end-product enhancement. Litwack and Rosenfield (1973) demonstrated a correlation between coenzyme dissociation and degradation rate constants for a number of enzymes.

A differential response to cytodifferentiation of the lysosomal and non-lysosomal components has been observed (Wilde *et al.*, 1980). Fatty acid synthetase degradation was partially inhibited by an inhibitor of the lysosomal component while an inhibition of casein degradation was not observed during the same time period.

A possible mechanism of degradative control has been proposed by Tischler (1980). Regulation of degradation in skeletal muscle may be mediated by changes in the cellular oxidation-reduction potential.

4. AN OVERVIEW OF LIPOGENESIS AND ITS RELATIONSHIPS TO OTHER METABOLIC PROCESSES OF THE LIVER.

A coherent picture of the interrelationships in liver metabolism is now emerging. Hepatic metabolism responds to a variety of stimuli and conditions (eg. diabetes, other hormonal imbalances, nutritional factors, genetically induced obesity, inflammation and other physiological trauma, etc.) in a coordinated fashion. Mechanistically the response involves controlling the levels of enzyme activities present in the cell under these varied conditions. Regulation can occur via short-term or long-term mechanisms which influence catalytic activity and absolute levels, respectively.

Some of the processes directly related to lipogenesis and β-oxidation are illustrated in Figure 8 (Geelen *et al.*, 1980). Under normal conditions when caloric intake exceeds the energetic needs of the body the metabolic flux of the liver is diverted to energy storage. The enzymes involved in glycolysis, gluconeogenesis and lipogenesis all increase in activity by both short- and long-term mechanisms. The latter involves long-term adaptive changes in enzyme activity due to fluctuations in the absolute amounts of key enzymes (Numa and Yamashita, 1974; Volpe and Vagelos, 1976). The other mechanism involves short-term modulation of enzyme activty by substrate supply, allosteric effectors, and covalent modifications through interconversion of enzymes between active and inactive forms by a phosphorylation-dephosphorylation cycle (Geelen *et al.*, 1980; Lane *et al.*, 1979, Hardie, 1981).

The key allosteric effectors influencing the lipogenic pathway, malonyl-CoA, long-chain acyl-CoA esters, acetyl-CoA, fructose-1,6-diphosphate and citrate (McGarry and Foster, 1980) are illustrated in

Figure 8: Summary of the metabolic pathways leading to lipid synthesis and breakdown (from Geelen *et al.*, 1980).

During the situation favoring glycogenesis, glycolysis and cholesterolgenesis (ie. high insulin, low glucagon) the flux of metabolites in the liver is to glycogen, fatty acids and cholesterol. The enzymes subject to covalent modulation are in the dephosphorylated mode under these circumstances. Allosteric effectors include fructose-1,6-diphosphate, which activates pyruvate kinase; pyruvate, which inhibits the kinase that inactivates pyruvate dehydrogenase; citrate, which may coordinate glycolysis and lipogenesis by inhibiting phosphofructokinase and activating acetyl-CoA carboxylase; and malonyl-CoA which inhibits carnitine acyltransferase 1 activity.

During the situation favoring glycogenolysis, ketogenesis and glucogenesis (low insulin and high glucagon) the flux of metabolites is to glucose and the ketone bodies. The enzymes subject to covalent modulation are in the phosphorylated mode and the allosteric effectors include acetyl-CoA which activates pyruvate carboxylase and long-chain acyl-CoA esters which inhibit acetyl-CoA carboxylase.

The enzymes described are also subject to long-term regulatory mechanisms.

The enzymes are numbered:

1. Glycogen synthase
2. Glycogen phosphorylase
3. Fructose biphosphatase
4. Phosphofructokinase
5. Pyruvate kinase
6. Pyruvate dehydrogenase
7. Citrate-malate antiport
8. ATP-citrate lyase
9. Acetyl-CoA carboxylase
10. β-hydroxy-β-methylglutaryl-CoA reductase
11. Fatty acid synthetase
12. Carnitine acyl-transferase
13. Glycerophosphate acyltransferase
14. Phosphatidate phosphorylase
15. Diacylglycerol acyltransferase

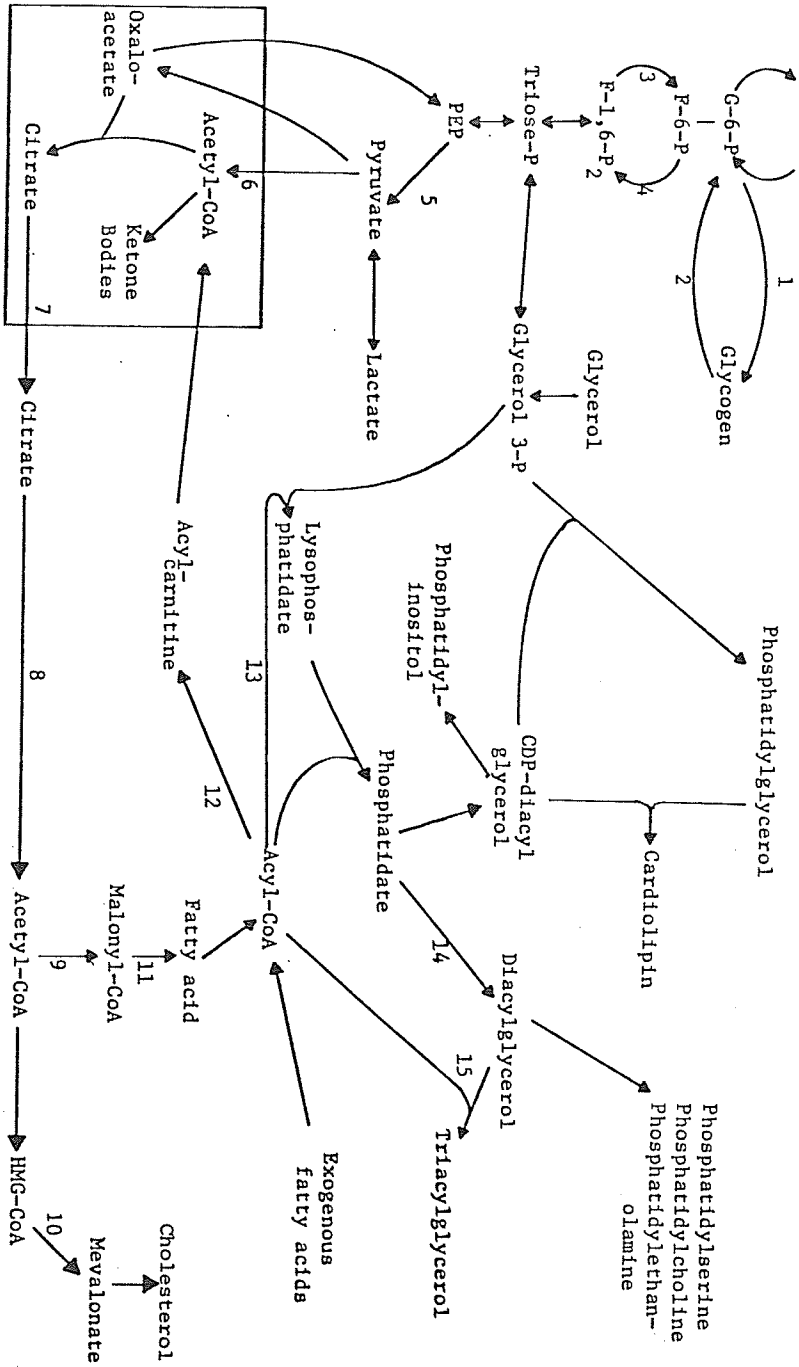

Glucose
G-6-P
F-6-P
F-1,6-P2
Glycogen
Triose-P
PEP
Pyruvate
Lactate
Glycerol
Glycerol 3-p
Oxalo-acetate
Acetyl-CoA
Citrate
Ketone Bodies
Citrate
Lysophos-phatidate
Acyl-carnitine
Phosphatidyl-inositol
CDP-diacyl glycerol
Phosphatidylglycerol
Cardiolipin
Phosphatidylserine
Phosphatidylcholine
Phosphatidylethan-olamine
Phosphatidate
Acyl-CoA
Diacylglycerol
Triacylglycerol
Exogenous fatty acids
Fatty acid
Malonyl-CoA
Acetyl-CoA
Cholesterol
Mevalonate
HMG-CoA
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 8. Also shown are the key regulatory enzymes subject to covalent modification. Under appropriate conditions the hepatic enzymes are in the dephosphorylated mode (i.e. low cyclic AMP, high insulin) which favors ketogenesis and gluconeogenesis (Hardie, 1981). A generalized observation is that enzymes involved in a biodegradative function are activated by phosphorylation whereas enzymes involved in biosynthesis are inactivated by phosphorylation (see Table 4; Krebs and Beavo, 1979; Cohen *et al.*, 1979).

This generalization is complicated by a multiplicity of regulatory mechanisms including cAMP-dependent protein kinases, Ca^{+} (calmodulin)-dependent protein kinases and other kinases; and by both multifunctional and specific phosphoprotein phosphatases. Phosphorylation-dephosphorylation appears to be one cellular mechanism used to synchronize the different pathways in the liver. The integration of hepatic metabolism appears to involve allosteric responses to variations in intracellular levels of effectors and covalent modification, in response to external stimuli, which can also modulate the allosteric controls (Hardie, 1981).

5. SHORT-TERM REGULATION OF FATTY ACID SYNTHESIS AND LIPOGENESIS

The acute response on short-term controls of lipogenesis in higher animals involves rapid activity changes in response to metabolic and hormonal effectors. Control is intimately involved with the control of carbohydrate metabolism with a fine balance drawn between storage of triglycerides and glycogen, utilization and production of blood-borne nutrients, production of membrane components and cellular energy needs (see Figure 8).

Table 4. Enzymes Found in the Cytoplasm of Mammalian Cells that are Regulated by Phosphorylation.

	Type of protein kinase			
	cAMP	Calmodulin	Other	
Activation by Phosphorylation				**Biodegradative pathway**
Glycogen Phosphorylase	-	+	-	glycogenolysis
Myosin	-	+	-	ATP hydrolysis
Phosphorylase kinase	+	+*	-	glycogenolysis
Triglyceride lipase	+	-	-	triglyceride breakdown
Cholesterol esterase	+	-	-	cholesterol ester hydrolysis
Inactivation by Phosphorylation				**Biosynthetic pathway**
Glycogen synthase	+	+	+	glycogen synthesis
Acetyl CoA carboxylase	+	+	+	fatty acid synthesis
Glycerol phosphate acyl transferase	+	-	-	triglyceride synthesis
HMG CoA reductase	-	-	+	cholesterol synthesis
Initiation factor eIF2	-	-	+	protein synthesis
L-type pyruvate kinase	+	-	-	gluconeogenesis

*Phosphorylase kinase phosphorylates itself.

Taken from Cohen *et al.*(1979).

5.1. Fatty Acid Synthetase

Wakil *et al.* (1966) reported allosteric regulation of FAS activity by phosphorylated sugars, especially fructose-1,6 diphosphate. This stimulation of FAS activity in pigeon liver and *E. coli* has also been reported for human liver FAS (Roncari, 1975). Kinetic analysis suggested the effect was related to relieving the inhibition of FAS by malonyl-CoA and decreasing the K_m of the enzyme for NADPH (Plate *et al.*, 1968). The significance of these studies appears questionable as the levels of sugars used were much higher than physiological levels and because other groups have failed to demonstrate the effect with purified FAS from pigeon or rat liver (Porter *et al.*, 1971) and rat mammary gland (Smith and Abrahan, 1970).

Long-chain fatty acyl CoA derivatives have an inhibitory effect on FAS although evidence indicates the effect may be non-specific and related to the ability of long-chain acyl CoAs to function as detergents (see Volpe and Vagelos, 1976).

Yu and Burton (1974a,b) have demonstrated the existence of an enzymatically inactive, immunologically reactive FAS molecule in rat liver during the early periods of refeeding after starvation. Crude preparations demonstrated increasing amounts of inactive enzyme from rats refed at 1, 2 and 3 hours. The authors also demonstrated generation of active FAS by incubating the extract with CoA, ATP and a 22-40% ammonium sulfate fraction from the livers of refed rats. Separation of the pigeon liver apo- and holo-enzyme forms has been achieved with affinity chromatography (Qureshi *et al.*, 1975). Roncari (1975) inactivated purified FAS with an extract from fasted rats which caused prosthetic group release. More recently a 4'-phosphopantetheine hydrolase enzyme has been partially purified from rat liver (Sobhy, 1979).

and its levels found to vary with nutritional status and increase in diabetic rats. Regulation of FAS activity may involve splitting the prosthetic group from the holoenzyme with the hydrolase and reformation with a transferase. Differential turnover of the two components may also be important as the 4'-phosphopantetheine turns over at a much faster rate (Tweto *et al.*, 1971; Liou and Donaldson, 1977).

CoA involvement in FAS regulation has recently been proposed (Lin *et al.*, 1980; Lin and Srere, 1980). The cytosolic concentration of CoA varies considerably (McCune and Harris, 1979) and it may be involved in the terminal reaction of fatty acid synthesis.

Phosphorylation-dephosphorylation has also been suggested as a mechanism of regulation (Qureshi *et al.*, 1975b) with the enzyme existing in phosphorylated holo-b (inactive) and dephosphorylated holo-a (active) forms. Other experimental evidence has implicated cAMP involvement and observed increases in the apo- and holo-b enzyme forms as fatty acid synthesis decreases. The importance of these results is questionable as other authors have been unable to confirm them. Glucagon incubation of rat hepatocytes did not lead to a decrease in enzyme activity (Witters *et al.*, 1979) nor to ^{32}P-labelling of the enzyme (Witters *et al.*, 1979b). Also, FAS contains only a limited amount of phosphate and the rate of phosphorylation is only 0.01% that of acetyl-CoA carboxylase (Hardie and Cohen, 1978).

Most evidence indicates short-term control of fatty acid synthesis is exerted primarily by acetyl-CoA carboxylase, the first enzyme in the pathway, and not by fatty acid synthetase. Coordinate regulation of FAS activity may occur through regulation of malonyl-CoA levels, the product of acetyl-CoA carboxylase (Cook *et al.*, 1977). Short-term incubations

of rat hepatocytes with glucagon (McGarry *et al.*, 1978) and insulin (Beynen *et al.*, 1979) confirms the proposal of short-term regulation of acetyl-CoA carboxylase and the role of malonyl-CoA levels in influencing FAS.

5.2. Acetyl-CoA Carboxylase and other Lipogenic Enzymes.

Short-term regulation occurs at a number of specific sites in the coordinated pathways of carbohydrate and lipid metabolisms. Recent and extensive reviews have covered the topic and only recent and interesting points will be covered here (see Geelen *et al.*, 1980; Beynen *et al.*, 1980; Zammit, 1981; Lane *et al.*, 1979).

Acetyl-CoA carboxylase is regulated by a variety of mechanisms including covalent modification, allosteric effectors and its state of aggregation. The enzyme from mammalian sources is active when its protomers combine into long, filamentous polymers. Aggregation is favoured by citrate while long chain acyl-CoA esters favor desegregation (Lane *et al.*, 1974). More recently Clarke and Hillard (1981) have found acetyl-CoA carboxylase activity is altered as fatty acids altered the degree of enzyme polymerization. The enzyme is also regulated by phosphorylation: dephosphorylation reactions catalyzed by cAMP-dependent protein kinases (i.e. glucagon and adrenalin stimulation) (see Geelen *et al.*, 1980). Inactivation by phosphorylation may lead to or occur concommitantly with enzyme depolymerization (Lent *et al.*, 1978). Recent observations indicate insulin activation may involve phosphorylation by a cAMP-independent protein kinase that acts at a separate site to the cAMP-dependent kinases, and may involve polymerization of the enzyme (Brownsey *et al.*, 1981). A regulatory protein that inhibits the enzyme by acting directly upon the carboxylase has also been isolated

(Abdel-Halm and Porter, 1980).

Phosphorylation of ATP-citrate lyase during glucagon incubation of rat hepatocytes has been observed although no correlation with enzyme activity changes occurred (Janski *et al.*, 1979). Besides this non-specific cAMP-dependent phosphorylation another cAMP-independent kinase specific for ATP-citrate lyase has been reported (Ramakrishna and Benjamin, 1981). As with acetyl-CoA carboxylase this phosphorylation occurs at a different site and is stimulated by insulin.

The enzymes in the glycolytic pathway are also subject to cAMP-dependent and independent phosphorylations-dephosphorylations (see Geelen *et al.*, 1980). This pathway as well as lipogenesis appears sensitive to the cellular energy change (ATP concentration) (see Attaullakaanov *et al.*, 1981) and to the cellular redox potential (see Berry, 1980).

β-hydroxy-β-methyl-glutaryl-CoA reductase, the first enzyme in cholesterol biosynthesis, is also regulated via the cAMP-dependent protein kinase system (see Geelan *et al.*, 1980). A number of studies have also implicated fluidity changes in microsomal membranes as regulating enzyme activity through changes in membrane cholesterol levels (Sipat and Sagine, 1981; Mirro Poulos *et al.*, 1981). Modulation (i.e. increasing activity by insulin treatment) is specific for HMG-CoA reductase and may, as in the case of acetyl-CoA carboxylase, involve a membrane-bound cAMP-independent kinase which influences the structural integrity of the membrane.

6. LONG-TERM REGULATION OF FATTY ACID SYNTHESIS AND LIPOGENESIS

Although there is little evidence implicating fatty acid synthetase in the short-term or acute control of fatty acid synthesis, there is considerable evidence that it is involved in long-term adaptive changes in enzyme quantity. Short-term regulation appears to involve both specific and general cellular mechanisms and responses to various stimuli. These short-term regulatory events may initiate or occur concommitantly with the initiation of long-term mechanisms and predictably the long-term mechanisms involve both generalized whole cell responses and group-specific responses.

Long-term adaptive changes have been observed for glucose-6-phosphate dehydrogenase (Sun and Holten, 1978), 6-phosphogluconate dehydrogenase (Hutchison and Holten, 1978), malic enzyme (Rudack *et al.*, 1972), citrate cleavage enzyme (Gibson *et al.*, 1972), acetyl-CoA carboxylase (Majerus and Kilburn, 1969), HM6-CoA reductase (see Geelen *et al.*, 1980) and fatty acid synthetase (Burton *et al.*, 1969) in response to nutritional, developmental, genetic and neoplastic factors. Coordinate long-term regulation is observed for these and most enzymes involved in lipogenesis although discrepancies in this generalization occur (see Volpe and Vagelos, 1976). Mechanistically, long-term changes in enzyme content involve changes in both the rate of synthesis and in the rate of protein degradation. The involvement of the various conditions affecting the levels of FAS and the changes in enzyme synthesis and degradation are presented in Table 5 (for references see Volpe and Vagelos, 1976 and text).

Table 5. Regulation of animal hepatic fatty acid synthetase

Condition	Specific Activity (U/mg protein)	Rates of Enzyme	
		Synthesis	Degradation
Nutritional[a]			
1. High-carbohydrate diet	↑[b]	↑	N
2. Fat-free diet	↓	↑	N
3. High-fat diet	↓	-	-
4. Polyunsaturated fatty acids	↓	-	-
5. Fasted	↓	↓	↑
6. Choline deprivation	↑	-	-
7. Vitamin B_{12} deprivation	↑	↑	±↑
Hormonal[c]			
1. Diabetes	↓	↓	-
2. Diabetes + insulin	N	N	-
3. Diabetes + fructose feeding	N	N	-
4. Glucagon	↓	↓	-
5. Theophylline	↓	↓	-
6. Dibutyryl cAMP	↓	-	-
7. Hyperthyroid	↑	-	-
8. Hypothyroid	↓	-	-
9. Hypophysectomized	↓	-	-
Development[d]			
1. Fetus	↓	-	-
2. Newborn	↓-N	-	-
3. Suckling	↓	↓	N
4. Weaned	↑	↑	N
Genetic (obese-hyperglycemic mouse)[e]			
1. Standard laboratory chow	↑	↑	N
2. Fasting	↑	↑	↓
3. Fasting, refed fat-free diet	↑	-	-
4. Triiodothyronine x 7 days	N	-	-
Neoplastic (minimal deviation hepatoma)[f]			
1. Standard laboratory chow	↓	-	-
2. Fasted	↑	-	-
3. Fasted, refed fat-free diet	↓	-	-

[a]Comparisons are between animals administered standard laboratory chow or indicated diets or factors. [b]N, ↑, and ↓: no difference from, greater than, less than control, respectively; -: no data available. [c]Comparisons are between animals subjected to indicated hormonal changes and normal control. [d]Comparisons are between adult animals and those of indicated ages. [e]Comparisons are between obese-hyperglycemic mice and normal mice subjected to indicated dietary alterations. [f]Comparisons are between neoplastic liver and normal liver for same animal subjected to indicated dietary alterations.

6.1 Nutritional Factors

Starvation results in a rapid decline in liver FAS levels (Allman *et al.*, 1965), while refeeding a fat-free high carbohydrate diet after 48 hours fasting results in a 10-50 fold increase in enzyme levels (Gibson *et al.*, 1966). These responses are most noticeable in the liver and adipose tissue where energy storage is a prominent function and less noticeable in the intestinal mucosa and not observed in the brain (see Volpe and Vagelos, 1976 for references). The nature of the sugar utilized in refeeding causes variations in the observed effects which differ from tissue to tissue. Fructose refeeding results in three-five fold higher increases in hepatic FAS than observed with glucose refeeding (Volpe and Vagelos, 1974). This effect is opposite to that observed in adipose tissue and may relate to the different functions of the two organs.

Feeding animals diets high in fat leads to a decrease in fatty acid synthesis and in FAS levels (Flick *et al.*, 1977; Wiegano *et al.*, 1973). Variations in the effect are observed with different types of fatty acids, different tissues and different animals.

Choline deficiency causes a rapid increase in FAS (Rosenfeld, 1973) and acetyl-CoA carboxylase (Chalvarojian, 1969) levels as does vitamin B_{12} deficiency (Frenkel *et al.*, 1974).

Nutritional regulation of metabolism is coordinated by the hormonal mechanisms of the body, although on a longer-term basis metabolite influences (possibly through secondary hormonal effects) may also function in regulation. Starvation lowers the levels of circulating insulin (Mayhew *et al.*, 1969) and thyroid hormones (Harris *et al.*, 1978) and increases the levels of growth hormone (Mayhew *et al.*, 1969)

and glucagon (Adibi *et al.*, 1976). Refeeding results in a rapid rise in serum insulin, and in glucagon output if the diet is high in protein and low in carbohydrate or a decrease in glucagon if the diet is high in carbohydrate and low in protein (Eisenstein and Strack, 1978). The insulin/glucagon balance appears to be one of the most important facets of dietary regulation, balancing the utilization of protein for energy in times of lowered food input with protein synthesis and energy storage under normal conditions (Fuller and Crofts, 1977).

Diabetic animals fail to induce the adaptive synthesis of FAS during refeeding, following starvation, unless insulin is administered (Lakshmanan *et al.*, 1972). Feeding a fatty diet decreases FAS levels but has no effect on insulin levels nor is prostoglandin synthesis required to observe FAS (Flick *et al.*, 1977). This suggests the involvement of other factors, although Kitajima *et al.* (1975) observed a reduction of acetyl-CoA carboxylase in a liver cell line in the absence of hormones.

The levels of growth hormone increase with refeeding but not as rapidly as insulin (Sukkar *et al.*, 1967) suggesting a different function for the hormone.

Mechanistically the nutritional control of FAS levels involves variations in the rates of synthesis and degradation (see Table 5). Burton *et al.* (1969) demonstrated that increased incorporation of label into purified hepatic FAS occurred with refeeding. Tweto and Larrabee (1972) observed that the lowest rate of synthesis in starved rats occurs after 16 h of fasting. Later studies, using isotopic-immunochemical techniques, determined the rate of constants of synthesis and degradation and found the decrease in FAS levels with starvation could be

accounted for by both decreases in synthesis and increases in degradation (Volpe *et al.*, 1973). Similar observations have been made for acetyl-CoA carboxylase (Majerus and Kilburn, 1969) and malic enzyme (Silpanta and Goodridge, 1971). Comparisons with fat-free fed animals and those fed a normal diet indicate the increase in FAS levels is due solely to an accelerated rate of synthesis. Discrepant results have been observed with vitamin B_{12} deficiency. Frenkel *et al.* (1974) found an increase in FAS activity with an increased rate of synthesis but also an increased rate of degradation. This result may indicate the existence of both general and specific mechanisms for controlling synthesis and degradation.

Evidence implicates both transcriptional, post-transcriptional and translational factors as important in the observed variations in FAS content. Long-term adaptions in the turnover of proteins may be modulated by ribosome content and distribution as was observed for secretable glycoproteins. During fasting ribosome content decreases rapidly during the first two days and at a slower rate thereafter if starvation is continued (see Munro, 1964). Animals growing at different rates due to nutritional limitations show a correlation between protein synthesis and ribosome content (Young *et al.*, 1971). Refeeding mice after a period of starvation results in a rapid increase in ribosome levels which is attributable to increased transcriptional activity and a decreased degradation of ribosomes (Conde and Franz-Fernandez, 1980). Original studies indicated that starvation or maintenance on protein-free diets for long-periods of time did not affect the relative proportions of free and membrane-bound ribosomes (Ramsey and Steele, 1976) but did have an effect on polysome size (Enwonwu *et al.*, 1971).

More recent studies (Shutler *et al.*, 1977; Neprokroeff *et al.*, 1979) indicate the increase in FAS activity correlates with an increase in the proportion of free to bound ribosomes.

The variations in ribosome levels observed under different conditions may not be important in the immediate long-term mechanisms but function over a longer period of time in stabilization of long-term controls. The variations in ribosome distributions may involve some of the initiation factors described previously. Kabat (1970) described a 60S ribosomal subunit protein which was phosphorylated only on monomeric ribosomes. This proposal has not been verified in other experiments (Leader and Coia, 1978).

Shutler *et al.* (1977) observed a 4-fold increase in the proportion of free/bound ribosomes while a 15-fold increase in activity is observed with 15 hours of refeeding a fat-free diet after starvation. Use of anti-synthetase antibodies to bind polysome-bound nascent chains indicated a 95-fold increase in chain synthesis for animals refed for 16 hours (Strauss *et al.*, 1975). More recent studies on fatty acid synthetase messenger RNA indicate refeeding a fat-free diet after starvation results in a large increase in translated product with a 25-fold increase in FAS activity (Flick *et al.*, 1978). Nepokroeff and Porter (1978) found that the adaptive synthesis of FAS could be largely accounted for by increased levels of messenger RNA.

Studies on the nutritional regulation of carbohydrate metabolism indicate a 5-6 fold increase in 6-phosphogluconate dehydrogenase, observed with refeeding, can be accounted for by a 5-6 fold increase in the levels of translatable message (Hutchison and Holten, 1978). In the case of glucose-6-phosphate dehydrogenase the 20 to 30 fold

increase in activity is accompanied by only a 2 to 3 fold increase in specific message (Sun and Holten, 1978). In both cases the induction of enzyme was due to an increased rate of synthesis implicating the involvement of both transcriptional and translational controls. The levels of another hepatic enzyme involved in carbohydrate metabolism, pyruvate kinase, also vary under different nutritional conditions and the change appears to involve mainly transcriptional controls of the levels of specific mRNA (Cladaras and Cottam, 1980).

Evidence from the general effects of fasting and refeeding implicate cytoplasmic factors as involved in translational control. In fasting rats the rates of translational initiation are decreased (Henshaw *et al.*, 1971; Fleck *et al.*, 1965). A similar observation has been made with Ehrlich cells deprived of glucose (van Venrooij *et al.*, 1972), the reversal of the observed inhibition of synthesis initiation occurs within minutes of refeeding with glucose.

6.2 Hormonal Factors.

Diabetic animals have markedly decreased levels of fatty acid synthetase, in both liver and adipose tissue, caused by a decreased rate of synthesis (Volpe and Vagelos, 1974; 1976), while administration of insulin increases the levels and rate of synthesis. The insulin effect has been observed for six other lipogenic enzymes suggesting a coordinate control of the rate of synthesis of these enzymes (Nepokroeff *et al.*, 1974). Fructose feeding to diabetic animals elicits a normal hepatic adaptive synthesis response suggesting the involvement of intermediates of glycolysis, at the triose-phosphate step or beyond, in the regulation of FAS biosynthesis (Volpe and Vagelos, 1974). The ability of fructose to mimic the effect of insulin is restricted to the

liver and not observed in adipose tissue (see Volpe and Vagelos, 1976).

Glucagon, also functions as a mediator of the levels of hepatic lipogenic enzymes (Volpe and Marasa, 1975). Adaptive synthesis of FAS is severely retarded if glucagon or dibutryl cyclic-AMP, is administered concurrently with a fat-free diet (Lakshmanan *et al.*, 1972). This lowering of hepatic FAS levels occurs with a decrease in the rate of enzyme synthesis. Studies with chick embryos indicate that cAMP, glucagon and insulin stimulate the synthesis of fatty acid synthetase (Joshi and Sidbury, 1976; Joshi and Wakil, 1978). The mechanism of glucagon stimulation may involve a marked increase in serum insulin levels after glucagon administration (Joshi and Aranda, 1979) as with liver explants from chick embryos no stimulation by glucagon was observed.

Triiodothyronine and thyroxine, the thyroid hormones, are found to enhance the levels of hepatic FAS (Kumar *et al.*, 1977) and acetyl-CoA carboxylase (Roncari and Murthy, 1975). Das (1980) suggests triiodothyronine functions in two stages. The first to enhance conversion of apo- to holo-enzyme in early long-term regulation and the second to increase the levels of FAS protein.

In adipose tissue hydrocortisone treatment inhibited the induction of lipogenic enzymes and this inhibition could be relieved by adrenalectomy (see Volpe and Marasa, 1975). Thus at least in peripheral tissue insulin and the glucocorticoids are believed to have antagonistic effects (Diamant and Shafrir, 1975). Adrenalectomy of diabetic animals relieves the inhibition of synthesis of lipogenic enzymes in adipose tissue although the effect may be modulated by glucocorticoid involvement in inhibition of glucose metabolism at the level of glucose transport (Munck, 1971). In hepatic tissue evidence indicates insulin

and the glucocorticoids act in concert during times of stress, although this effect may be specific for the enzymes involved in fatty acid synthesis (Diamant and Shafrir, 1975). Berdanier and Shubeck (1979) found that rats stressed by starvation and refeeding required the presence of both hormones to observe the typical increase in enzyme levels. These authors observed the same effect in genetically obese mice but in normal (non-stressed) animals no requirement for glucocorticoids was observed. The level of interaction of the different hormones is unknown as diabetic rats produce less glucocorticoids than non-diabetic animals (De Nicola *et al.*, 1977) and the increases in glucocorticoids inhibit the release of the thyroid hormones. Induced-diabetic rats also have an impaired ability to convert thyroxine into the more active form, thiiodothyranine (Balsam and Ingbar, 1972).

Diabetes is found to reduce the protein-synthesizing activity of ribosomes in skeletal muscle (Wool, 1972) and in liver (Morgan and Peters, 1971). The impairment appears to be mediated by an inhibition of translation initiation (Pain, 1973) although other mechanisms have been suggested (see Roy *et al.*, 1980). The possibility arises that selective changes in message degradation caused by diabetes may be involved implicating cytoplasmic factors (possibly association proteins) (Rosen *et al.*, 1980). Insulin has been reported as increasing protein synthesis by a stimulation of the amount of mRNA being translated by the ribosomes (Fahmy and Leader, 1980). Evidence implies this induction may involve activation of pre-existing message rather than a transcriptional increase although attempts to elucidate the mechanism have been unsuccessful (Leader *et al.*, 1978).

Other evidence indicates insulin, triiodothyronine and hydrocortisone stimulated increases in cultured explants and chick embryos involves

an increase in functional nuclear message (Ross *et al.*, 1977; Joshi and Aranda, 1979). Experiments with triiodothyronine and refeeding a fat-free diet indicate the increase in lipogenic enzymes is accompanied by an increase in messenger RNA levels (Towle *et al.*, 1979; Mariash *et al.*, 1980). Steroid hormones have been implicated in many studies as having a direct influence on transcriptional rates (see discussion on transcriptional regulation and Baxter *et al.*, 1972).

Studies on protein degradation during hormonal changes have given inconclusive results (for review see Goldberg and St. John, 1976). The current view is that degradative changes occur in response to hormonal variations but their importance is less than the observed variations in the rate of synthesis. Dice *et al.* (1978) reported changes in the rate of protein degradation in muscle and liver after insulin removal from diabetic rats. Glucagon causes a rapid increase in the rate of proteolysis in liver (Woodside *et al.*, 1974) which may involve the appearance of autophagic vacuoles in liver and an increase in the fragility of lysosomes (Mortimore and Nealy, 1975). Recent experiments indicate glucagon, acting via cyclic-AMP, increases proteolysis in hepatocyte monolayers. This response is inhibited by insulin which acts via a different mechanism involving a direct interaction with the nucleus (Hopgood *et al.*, 1980). Insulin involvement with degradative rates has also been observed with other inducible enzymes. Hepatic levels of phosphofructokinase decrease during diabetes primarily due to an accelerated rate of degradation (Dunway *et al.*, 1978). The induction of tyrosine aminotransferase with insulin is thought to involve a selective slowing of its degradation (Spencey *et al.*, 1978) while glutathione-insulin transhydrogenase stimulation by insulin

involves increases in the rate of synthesis (Hern and Varandani, 1980). This last effect may implicate another mechanism as insulin is substrate of the enzyme.

6.3 Other Factors

Developmental influences on the adaptive synthesis of FAS and acetyl-CoA carboxylase have been observed under a variety of conditions (see Volpe and Vagelos, 1976). Synthetase activity rises dramatically in avian liver after hatching and mammalian liver transiently at birth and then permanently after weaning (Volpe and Kishimoto, 1973). The molecular basis involves an increased rate of synthesis and a constant rate of degradation during periods of enzyme increase.

Recent studies on mammalian cells in culture indicate an increased rate of lipogenesis and a coordinate rise in the lipogenic enzymes during differentiation. The observed increase during differentiation of 3T3-L_1 preapidocytes into apidocytes is attributed to an increased rate of synthesis (Student *et al.*, 1980). Treatment of developing apidocytes with adrenocorticotropic hormone isoproterenol and dibutyryl cyclic-AMP caused a decreased rate of synthesis implicating hormonal involvement in developmental expression of FAS (Weiss *et al.*, 1980). Other studies with mammary gland explants indicate both an increased rate of synthesis and a lowered rate of degradation occur during differentiation (Mayer and Paskin, 1978). A two-component degradative system has been identified in these cells with differential effects observed for FAS and casein, a secretable protein (Wilde *et al.*, 1980). Degradation rates in rat liver during post-natal development are half those found in the adult animal and some heterogeneity of degradation rates for different groups of proteins is observed (Russell *et al.*,

1980).

Genetically obese-hyperglycemic mice have regulatory changes in their controls of fatty acid synthesis (see Volpe and Vagelos, 1976). The observation that starvation causes a decrease in FAS synthesis in both normal and obese animals but an increased degradation only in the non-obese animals may imply a control existing between synthesis and degradation in normal animals (see Volpe and Marasa, 1975b). Other studies with lipemic rats suggest that other factors than increased amounts of lipogenic enzymes must be involved in the higher rate of fatty acid synthesis (Lakshmanan *et al.*, 1977).

EXPERIMENTAL PROCEDURES

1. MATERIALS

Male, Long-Evans (hooded) rats, 150-200 g, were purchased either from Canadian Breeding Farms, St. Constance, Quebec or from the Central Animal Care Services, University of Manitoba, Winnipeg, Manitoba.

Radiochemical compounds were purchased from Amersham Corporation. The rabbit reticulocyte lysate translation kit containing L-[3,4,5-^{3}H(N)] leucine was purchased from New England Nuclear Corporation.

Acyl-CoA esters, NADPH, dithiothreitol and the ribosomal RNA standards (*E. coli* and calf-liver) were purchased from P-L Biochemicals, Inc. Diethyl pyrocarbonate, heparin, Coenzyme A and calf-liver transfer RNA were purchased from Sigma Chemical Co.

Oligo(dT)-cellulose, RNAse-free sucrose, goat anti-rabbit globulin antiserum were purchased from Bethesda Research Laboratories, I.C.N. Biochemicals and Difco Laboratories, respectively.

Other chemicals were obtained from various commercial sources, and were of analytical grade.

2. METHODS

2.1. Treatment of Animals

The rats were maintained on laboratory chow (Victor Fox Foods, Winnipeg). In most experiments, rats were starved for 48 hours and then refed a fat-free high-carbohydrate diet (Nutritional Biochemicals Div., I.C.N. Ltd.) as indicated. At appropriate times after refeeding (see text) a subcutaneous injection of oil of turpentine (0.5 mL/100 g body weight) was administered, under ether anesthesia, in the dorsolumbar region (Maung *et al.*, 1968). Control animals were maintained on a normal diet or received injections of 0.15 M NaCl instead of turpentine. Rats were killed at various times, as indicated in the text, by cervical

dislocation and their livers removed onto ice. Protocols for individual experiments are given in the text.

2.2 Physical and Chemical Methods

Protein concentration was determined either by the method of Gornall *et al.* (1949) or by the method of Lowry *et al.* (1951).

Spectrophotometric measurements were performed on a Gilford SP2400 Recording Spectrophotometer.

Measurements of radioactivity were obtained from a LKB Wallac 1215 Rackbeta or a Beckman LS230 liquid scintillation counter. The scintillation fluids used for radioactive measurements were: Aquasol-2 (New England Nuclear Corp.) for measurements of radioactivity in the polyacrylamide gel slices, Brays solution (containing 60 g Napthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol, 20 ml ethylene glycol and dioxane to one litre) and Scintiverse (Fisher Scientific Co.) for the other measurements.

2.3 Purification of Rat Liver Fatty Acid Synthetase

Two different procedures were utilized over the course of this research. Both yielded enzyme preparations that were similar in activity and quantity.

I: Rat liver supernatant solutions were prepared by the method of Wakil *et al.* (1957) as modified by Hsu *et al.* (1965). Livers from rats fed a fat-free diet for varying times, following starvation, were homogenized (3 x 20 second bursts at medium speed with a Polytron (Brinkman Instruments) or a Sorval Omnimixer) in 1½ volumes (v/w) of a buffer containing 70 mM potassium bicarbonate, 85 mM dibasic potassium phosphate and 9 mM monobasic potassium phosphate, pH 8.0.

The crude homogenate was centrifuged at 11,000 g (av) for 15 minutes and the pellet discarded. The supernatant was spun at 105,000 g (av) for 60 min and the microsomal pellet discarded. The rat liver 105,000 g supernatant was stored at -20°C under N_2 gas.

Rat liver fatty acid synthetase was prepared by the procedure of Burton *et al.* (1968) as modified by Nepokroeff *et al.* (1975). Frozen rat liver supernatant solution (40 ml) was allowed to thaw at room temperature. Saturated ammonium sulfate solution, pH 7.0 containing 3 mM EDTA and 1 mM β-mercaptoethanol, was added to a saturation of 20%. After stirring for 15 min (under N_2 gas), the mixture was centrifuged at 20,000 g for 15 min and the pellet discarded. The supernatant solution was brought to 33% saturation with ammonium sulfate and the precipitated protein collected by centrifugation. The pellet was dissolved in approximately 25 ml of a 5 mM potassium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM dithiothreitol (DTT), and treated with calcium phosphate gel as described by Hsu *et al.* (1965). The supernatant solution obtained from the calcium phosphate gel treatment was adsorbed onto a column of DEAE-cellulose (10.3 cm x 3.5 cm) which was previously equilibrated with a 50 mM potassium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM DTT. The column was washed with the 50 mM potassium phosphate buffer until the protein absorbance at 280 nm fell to below 0.05 units. The enzyme was eluted from the column with 0.16 M potassium phosphate, pH 7.0 containing 3 mM EDTA and 1 mM DTT, and a 0-33% ammonium sulfate precipitation was performed on the eluted fractions containing the A_{280} protein peak. The pellet was dissolved in a minimum volume of 0.5 M potassium phosphate buffer, pH 7.0 containing 3 mM EDTA and 5 mM DTT, and dialyzed overnight

against the same buffer. The purified rat liver FAS was stored at -20°C and was stable for at least one month.

All operations except the dialysis and centrifugations (4°C) were carried out at room temperature.

II: The second method was a modification of the procedure outlined by Stoops *et al.* (1979). Rats were fasted for two days and refed a fat-free high-carbohydrate diet as indicated. The livers were excised from rats (killed by cervical dislocation), washed with 0.15 M NaCl and homogenized in 1 volume (v/w) of 0.05 M potassium phosphate, pH 7.4 containing 2 mM DTT and 1 mM EDTA. The homogenate was centrifuged at 105,000 g (av) for 1 hour and the supernatant passed through glass wool and applied to a DEAE-cellulose column (2.8 x 35 cm) equilibrated with homogenization buffer. The supernatant was diluted 5-fold as it was applied to the column and the column was washed with the 0.05 M buffer until the protein absorbance at 280 nm was less than 0.25. The enzyme was eluted with 0.16 M potassium phosphate, pH 7.4 containing 2 mM DTT and 1 mM EDTA, or with a linear gradient consisting of equal volumes of homogenization buffer and 0.25 M potassium phosphate, pH 7.4 containing 2 mM DTT and 1 mM EDTA. Fractions in the protein peak (with a specific activity greater than 200 nanomoles NADPH oxidized/minute/mg protein) were pooled and subjected to a 0-33% ammonium sulfate precipitation. The pellet was dissolved in 7-10 ml (for 50-100 g liver) of homogenization buffer and applied to a Sepharose 4B column (2.5 x 35 cm) equilibrated with, and eluted with 0.5 M potassium phosphate, pH 7.4 containing 5 mM DTT and 1 mM EDTA. Fractions in the protein peak with a specific activity above 500 units/mg were pooled and the protein precipitated between 0-40% of saturation with ammonium sulfate.

The pellet was gently dissolved in 0.5 M potassium phosphate, pH 7.4 containing 5 mM DTT and 1 mM EDTA, and dialyzed overnight against the same buffer. The purified enzyme was stored at -20°C.

All operations were carried out at 4°C.

2.4 Assay for Fatty Acid Synthetase Activity

Rat liver fatty acid synthetase can be assayed by either radiochemical or spectrophotometric means. Prior to either procedure, preincubation of the enzyme solution at 37°C for 30 minutes, in the presence of 5 mM DTT, was performed.

The radiochemical procedure measured the incorporation of [1-^{14}C] acetyl-CoA into long-chain fatty acids outlined by Hsu *et al.* (1965) and modified by Butterworth *et al.* (1966). The assay mixture contained 15 nmoles [1-^{14}C]acetyl-CoA (specific activity 3.7 nCi/nanomole), 60 nmoles malonyl-CoA, 100 nmoles NADPH, 3 µmoles EDTA, 1 µmole dithiothreitol, 200 µmoles potassium phosphate buffer, pH 7.0 and enzyme in a final volume of 1 ml. The reaction was started by the addition of enzyme and allowed to continue for 6 minutes at 37°C. The reaction was stopped by the addition of 30 µl of 60% perchloric acid. One millilitre of ethanol was added to each assay mixture and the ^{14}C-labelled fatty acids were extracted 3 times with 2 ml of petroleum ether. The pooled petroleum ether extracts were evaporated to dryness in a counting vial, and radioactivity determined following addition of scintillation fluid.

The spectrophotometric assay measured the malonyl-CoA and acetyl-CoA dependent rate of NADPH oxidation as outlined by Collins *et al.* (1971) and modified by Nepokroeff *et al.* (1975). The assay mixture contained 15 nmoles acetyl-CoA, 60 nmoles malonyl-CoA, 5 µmoles DTT,

3 µmoles EDTA, 200 µmoles potassium phosphate buffer, pH 7.0 and enzyme (5-10 µg of DEAE-cellulose purified FAS or 50-100 µg of 105,000 g supernatant) in a final volume of 1 ml. The reaction was initiated by addition of enzyme to the mixture of substrates previously equilibrated at room temperature for 5 min. The oxidation of NADPH was followed at 340 nm, with the initial slope of the recorder tracing used to calculate the rate of fatty acid synthesis. A correction was made for the rate of NADPH oxidation in the absence of malonyl-CoA.

A unit of enzymatic activity is defined as the amount of enzyme required to catalyze the formation of 1 nanomole palmitic acid (or the oxidation of 14 nanomoles of NADPH) per minute under the conditions of the assay. The specific activity is defined as the number of activity units per milligram of protein.

2.5 Preparation of Antiserum

Purified FAS preparations were analyzed by analytical ultracentrifugation with a Spinco Model E ultracentrifuge, sucrose density gradient centrifugation as described by Martin and Ames (1961) and gel electrophoresis essentially as described by Laemmli (1970) and judged homogeneous.

Rabbit antiserum to rat liver fatty acid synthetase was prepared essentially by the method of Simkin *et al.* (1964) as previously described by Yu and Burton (1974). An intramuscular injection of 0.75 mg enzyme was followed 6 days later by an injection of 1.5 mg enzyme, both as emulsions of purified enzyme in 0.15 M NaCl and Freunds complete adjuvant. After a further 22 days, 3 intravenous injections of 0.4 mg, 0.75 mg and 1.9 mg of enzyme prepared as a coprecipitate with aluminium hydroxide were administered at 2 day intervals.

The animal was bled 5 days after the last intravenous injection. The blood serum obtained was subjected to ammonium sulfate fractionation as described by Volpe *et al.* (1973). The protein precipitating between 0 and 40% saturation was dissolved in ½ volume of 40 mM potassium phosphate, pH 7.0 containing 0.15 M NaCl and 1 mM DTT, dialyzed overnight against the same buffer and stored in aliquots at -20°C.

2.6 Immunochemical Procedures

The immunological homogeneity of the antisera was tested by the double diffusion method of Ouchterlony (1953) and showed only a single precipitin band for antiserum prepared against purified FAS.

Immunochemical titrations of FAS preparations were performed essentially as described by Kabat and Meyer (1961). In the first series of experiments, varying amounts of enzyme (DEAE-purified and 105,000 g supernatant) were added to a constant quantity of partially purified anti-FAS serum. In the second series of experiments, immunochemical equivalence points were determined by adding varying amounts of partially purified anti-FAS serum (up to 220 µg) to a constant quantity of 105,000 g liver supernatant prepared as described. A volume of liver supernatant containing approximately 5 units of FAS activity was used regardless of the specific activity of the preparation. For both techniques, the mixtures were incubated for 30 min at 37°C, clarified by centrifugation and the supernatants assayed for FAS activity. The data was plotted as a percentage of original activity remaining versus increasing antibody or enzyme concentrations. Extrapolation of the linear portion of the curve to 100% inhibition was taken as indicating the amount of antibody required for complete titration.

Material cross-reacting with anti-FAS serum, presumably FAS, was quantitated in the 105,000 g supernatant by a radial diffusion method based on that of Mancini *et al.* (1964) as modified by Burton *et al.* (1979). Gels of 2 mm thickness and containing 1% agarose, 1% rabbit antiserum against FAS, 0.5 mM EDTA and 0.25 M potassium phosphate buffer, pH 7.0, were prepared on glass plates measuring 20 x 20 cm. Wells 2.5 mm in diameter are cut in the gel. Aliquots of 5 µl of each 105,000 g supernatant solution (undiluted and diluted as indicated up to a 1/10 dilution), containing known quantities of enzyme activity, were placed in each well and diffusion was allowed to continue at room temperature for up to 4 days. The gels were immersed for 1-2 days in 0.15 M NaCl to remove soluble proteins and then washed with deionized water. The diameter of the precipitin rings formed could be measured immediately, or after staining with 0.1% amido black in 0.55 M acetate buffer, pH 3.75. The diameter of the precipitin ring bore a linear relationship to the logarithm of the amount of FAS used over a range of 1-10 µg of purified FAS.

The effects of varying the ionic strength of the potassium phosphate buffer and the inclusion of dithiothreitol in the gel were also tested.

2.7 Determination of the Relative Rates of Synthesis of FAS

Male, Long-Evans rats were fasted for 48 h and refed a fat-free diet for periods of 0 to 162 h. Animals to be inflamed were given a subcutaneous injection of oil of turpentine (0.5 mL/100 g body weight) at 0 h or 62 h refeeding. One hour prior to killing, each rat received a single intraperitoneal injection of L-[U-^{14}C]leucine (10 µCi/rat) and was killed at the specified time after refeeding.

The excised liver was homogenized and the 105,000 g supernatant prepared as described by Lakshmanan *et al.* (1975). Radioactivity incorporated into total soluble protein was determined by trichloroacetic acid precipitation of aliquots from the 105,000 g supernatant solution. Immunochemical precipitations of FAS were done by two methods which gave essentially equivalent results. The first method was described by Volpe *et al.* (1973) and involved a 20-40% ammonium sulfate fractionation of the 105,000 g supernatant solution prior to the addition of the antisera. The enzyme solution was assayed for FAS activity and enough rabbit anti-FAS serum added to precipitate 1½ times the units of FAS present. The second method was that of Nepokroeff and Porter (1978) and involved direct immunoprecipitation of the 105,000 g supernatant. The precipitate was resuspended in buffer, washed by pelleting through a discontinuous sucrose gradient followed by two washes with 0.15 M NaCl and dissolved in sodium hydroxide or formic acid for determination of incorporated radioactivity.

Control immunoprecipitations with non-immune sera served as a test for non-precipitation while the coprecipitation technique of Schimke (1965) was used to test for co-precipitation of label. The relative rate of synthesis of FAS is the ratio of radioactivity found in the immunoprecipitates to that incorporated into total protein.

2.8 Determination of the Relative Rates of Degradation and the Turnover Index of FAS.

Relative rates of degradation were determined by an isotopic-immunochemical technique similar to that used to determine the relative rates of synthesis (Schimke and Doyle, 1970). The rats were starved for 48 h and refed a fat-free diet for periods of 0 to 162 h.

Animals to be inflamed were given a subcutaneous injection of oil of turpentine (0.5 ml/100 g body weight) at 0 h or 62 h refeeding. The livers were excised and the 105,000 g supernatants treated as described for determination of the relative rates of synthesis.

L-[4,5-^{3}H]leucine (decaying label) was administered to each rat (0.25 µCi/rat) at the beginning of each experiment, prior to the start of starvation or at 62 h refeeding. The animals were killed at specified times, up to four days after injection of the decaying label, and the incorporation of radioactivity in FAS and total soluble protein was measured as described (Volpe *et al.*, 1973; Arias *et al.*, 1969). The protein half-life, or the time required for one-half the amount of decaying label to be lost from the specified protein or proteins, was determined by plotting log of ^{3}H-leucine DPMs incorporated versus time after injection.

Each animal also received an injection of L[U-^{14}C] leucine (non-decaying label) one hour prior to killing which allowed determination of the turnover and turnover-index of FAS and the total soluble protein (Ganschow and Schimke, 1969; Ciaranello and Axelrod, 1973). The turnover was the ratio of radioactivity found in immunoprecipitated FAS from the non-decaying label to that from the decaying label. The turnover-index was the ratio of the turnover for FAS to the turnover for total soluble protein.

2.9 Preparation and Isolation of Messenger RNA

Total cytoplasmic, magnesium precipitable RNA was isolated by the procedure of Palmiter (1974).

To minimize the danger of both exogenous and endogenous ribonuclease activity a number of precautions were routinely employed. All

buffers were sterilized and treated with 0.05% diethylpyrocarbonate to destroy residual RNAse activity. After addition of diethylpyrocarbonate, the buffer was shaken vigorously at room temperature and placed in a boiling water bath for 15 to 30 min, to decompose the remaining diethylpyrocarbonate. While still hot, the solutions were shaken vigorously to allow CO_2 and ethanol escape. All glassware was acid washed, sterilized by autoclaving and rinsed with 0.1% diethylpyrocarbonate prior to use. Disposable latex gloves were worn at all times and unless otherwise stated all procedures were performed at 0-4°C. Heparin, another ribonuclease inhibitor, was also included in the buffers as indicated.

Buffers were prepared from a stock solution containing 0.25 M Tris, 0.25 M NaCl, and 0.05 M $MgCl_2$, pH 7.5. Buffer A contained 10% stock buffer, 1 mg/ml heparin and 2% Triton X-100. Buffer B contained 4 volumes buffer A and 1 volume 1.0 M $MgCl_2$. Buffer C contained 10% stock solution, 0.2 M Sucrose (RNAse-free) and 0.2 mg/ml heparin.

Male, Long-Evans rats were starved for 48 h and refed a fat-free diet for the times indicated. Inflammation was induced at 0 h refeeding by a subcutaneous injection of oil of turpentine as described previously. Excised livers were placed immediately in ice-cold buffer A. A 10% homogenate was prepared by mincing liver samples in 9 volumes (v/w) buffer A and homogenizing in a Potter-Elvehjem homogenizer with 10 strokes at medium speed with a tightly fitting pestle. The homogenate was centrifuged at 27,000 g (av) for 5 min and the pellet discarded. The supernatant was mixed with an equal volume of buffer B and incubated at 0°C for at least 1 h. The precipitate was collected by layering over ½ volume buffer C and centrifuging for 10

min at 27,000 g. The supernatant was removed by aspiration part way into the sucrose layer and the upper portion of the tube was washed with distilled water. The wash was removed by aspiration and the tube was inverted to decant the remaining sucrose. The sides of the tube were wiped dry and the pellet dissolved in 20 mM Hepes, pH 7.5 (4.5 ml/g liver).

The RNA sample in 20 mM Hepes was diluted 1:1 with 0.1 M sodium acetate, pH 5.0 containing 1% SDS and the A_{260}/A_{280} ratio was determined (all subsequent steps were performed at room temperature to keep the SDS in solution). The RNA in this solution was extracted by a phenol: chloroform procedure as outlined by Palmiter (1974). The aqueous phase was collected and the RNA precipitated with two volumes of ice-cold ethanol. After twelve hours at -20°C the RNA was collected by centrifugation (27,000 g for 10 min) and washed twice with 3.0 M sodium acetate, pH 6.0. The RNA was dissolved in 0.1 M sodium acetate, pH 7.0, and precipitated overnight with ethanol at -20°C. The pellet was collected, dried under N_2 and dissolved in 0.1 M sodium acetate, pH 7.0, and precipitated overnight with ethanol at -20°C. The pellet was collected, dried under N_2 and dissolved in high salt application buffer, 10 mM Tris, 500 mM potassium chloride, pH 7.5 (2.0 ml buffer/ g liver) and A_{260}/A_{280} readings were made.

Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography as described by Aviv and Leader (1972) and modified by Nepokroeff and Porter (1978). RNA in the high salt application buffer, with an A_{260}/A_{280} ratio of approximately 2.0 and a concentration of 1 mg/ml (1 A_{260} unit represents 50 µg of RNA, Haines *et al.*, 1974) was applied to an oligo(dT)-cellulose column (5 mg RNA/g oligo(dT)-

cellulose) previously equilibrated with 20 volumes of the high salt buffer. The sample was washed through with a small amount of buffer and applied to the column again. The column was washed with the high salt buffer until the A_{260} was less than 0.04. The RNA bound to the column, i.e., the poly(A)-containing RNA, was eluted with a low salt buffer, 10 mM Tris, pH 7.5. The poly(A)-rich RNA was precipitated overnight with ethanol at -20°C, collected by centrifugation and washed twice with 95% ethanol. The final pellet was dried under N_2 and dissolved in 20 mM Hepes, pH 7.5, or in distilled water. The poly(A)-RNA collected was approximately 2-4% of the RNA applied to the column and had an A_{260}/A_{280} ratio of 2.0 to 2.2.

Poly(A)-RNA from membrane-free polysomes was isolated by a method modified from the combined procedures of Ramsey and Steele (1976b) and Palmiter (1974) as described by Lau *et al.* (1979). Livers were homogenized in 3 volumes of polysome buffer (25 mM Tris, 25 mM NaCl, 5 mM $MgCl_2$, 100 µg/ml heparin, pH 7.5). The homogenate was centrifuged at 2000 rpm for 2 min and then at 95,000 g (av) for 12 min (SW 27 rotor). The supernatant was diluted with an equal volume of polysome buffer, containing 200 mM $MgCl_2$, 2% Triton X-100 and 1 mg/ml heparin, and incubated at 0°C for at least 1 h. The precipitate was collected by centrifugation through ½ volume sucrose buffer as described previously. The rest of the procedure was identical to that described for isolation of total poly(A)-containing mRNA.

2.10 Characterization and Analysis of Poly(A)-containing RNA.

Sucrose density gradient centrifugation was performed as described by Lau *et al.* (1979). The poly(A)-containing RNA was heat treated in low salt solution to eliminate aggregation as reported by Haines *et al.*

(1974). The heat-treated RNA was fractionated by sedimentation through a 5-20% linear sucrose gradient in 0.1 M sodium acetate, pH 5.5 containing 1.0 M EDTA and 0.5% SDS. Centrifugation was at 20°C for 4 h at 40,000 rpm in an SW 41 rotor. The RNA distribution was monitored at 260 nm. Regions of the gradient were pooled and the RNA precipitated with ethanol, washed with 3.0 M sodium acetate and 95% ethanol and dissolved in distilled water.

Linear sucrose gradients (10-25%) in 10 mM HEPES, pH 7.5, containing 1 mM NaCl were also performed (Nepokroeff and Porter, 1978). Centrifugation was at 25,000 rpm for 24 h at 4°C (SW41 rotor). Purified ribosomal RNA (calf liver and *E. coli*) from P-L Biochemicals was used as a standard for size analysis.

Characterization of poly(A)-containing RNA was also accomplished by electrophoresis on 1.5% agarose gels containing 6.0 M urea and 0.025 M citric acid, pH 3.5 (Rosen *et al.*, 1975). Electrophoresis-grade agarose (Sigma) was gently dissolved in the urea buffer (Ultrapure urea, Schwartz-Mann) and poured either in 5 mm (i.d.) tubes or in a slab gel apparatus. RNA was dissolved in 6.0 M urea, 0.025 M citric acid, pH 3.5 buffer containing glycerol and 0.05% bromophenol blue. Electrophoresis was at 4°C at 2-3 mAmps/tube. The RNA bands were visualized by staining with ethidium bromide. The gels were first washed with cold distilled water and then stained for 30 min with 5.0 µg/ml ethidium bromide in 0.05 M Tris, pH 8.0. After rinsing with water the bands were illuminated using a short-wave ultraviolet lamp. Ribosomal RNA standards were also run for size comparisons.

2.11 Translation of Poly(A)-mRNA in a Reticulocyte Lysate Cell-Free System.

In vitro translation experiments were performed with a nuclease-treated rabbit reticulocyte lysate translation system from New England Nuclear produced by a modification of the method of Pelham and Jackson (1976). Unless otherwise indicated each assay mixture contained 80 mM K^{+} (potassium acetate), 1.0 mM Mg^{+2} (magnesium acetate), 17 µCi L-[3, 4,5-^{3}H(N)] Leucine (145 Ci/mmol), 10 µl of rabbit reticulocyte lysate, 2.0 µl of premixed translation cocktail, and RNA, in a total volume of 25 µl.

The lysate had been treated with a calcium activated nuclease, to remove endogenous message, and the enzymatic reaction was terminated with EGTA. Hemin was also added to the lysate. The translation cocktail contained all the components necessary for translation including spermidine, creatine phosphate and guanosine triphosphate in HEPES buffer.

Preparations of poly(A)-containing RNA dissolved in distilled water, usually 1.0 µg in 1.0 to 2.0 µl, were pipetted into 1.8 ml Eppendorf tubes on ice. A volume of 13 µl premix (all the above ingredients except the lysate) was added; followed by 10 µl of reticulocyte lysate; the mixture was quickly vortexed and centrifuged briefly in an Eppendorf centrifuge. After incubating in a 37°C water-bath for 1 h, the reaction was stopped by addition of 25 µl of 6.0 mM methionine and the tubes were placed on ice.

Conditions of the assay were varied to test for optimization of the N.E.N. Translation Kit, for rat liver FAS mRNA translation, as described in the text.

2.12 Translation Product Analysis.

Total protein synthesis was determined by pipetting 5 µl of sample onto a small square of Whatman 3 mm filter paper pretreated with TCA, according to the method of McLeester and Hall (1977). The sample precipitated on the filter paper was washed with cold 10% TCA, boiling 10% TCA, cold 10% TCA, ethanol, acetone and air-dried prior to counting for incorporated radioactivity.

Immunoprecipitation of material cross-reacting with rabbit antiserum to FAS was performed by two methods, the single and double antibody techniques (outlined by Comstock *et al.* 1981). The remainder of the translation solution was diluted to 0.45 ml with 0.15 M NaCl, 1.0 mM sodium azide and 4.7% Dextran T-70 as described by Jamieson *et al.* (1972). For the double antibody technique (Nepokroeff and Porter, 1978) the diluted translation solution was incubated with a volume of rabbit anti-FAS serum for 1 h at 37°C, followed by 12 h at 4°C. Sufficient goat anti-rabbit globulin antiserum was then added for quantitative precipitation and the solution incubated for 1 h at 37°C and 24 h at 0°C. Quantitative precipitation for the two antisera was determined prior to use and the same ratio was used in each experiment. The precipitate was collected by centrifugation and washed either by pelleting through sucrose and detergent (Shapiro *et al.*, 1974) or as described by Jamieson *et al.* (1972b).

The single antibody technique involved the addition of purified carrier FAS and sufficient rabbit anti-FAS antiserum to precipitate 1½ times the amount of purified FAS added. The solution was incubated for 1 h at 37°C, followed by 24-48 h at 4°C and collected as described above.

Co-precipitation and non-specific precipitation was tested, as described previously, in both methods. The immunoprecipitate was either dissolved in 100 µl of 0.1 N NaOH and counted for incorporated radioactivity or was dissolved in 50 µl of 0.01 M sodium phosphate, pH 6.8 containing 2% SDS and 2% β-mercaptoethanol (solubilized by heating to 90°C for 3 min) and used for electrophoresis.

Electrophoresis of the immunoprecipitable translation products was performed by the SDS-polyacrylamide gel method of Weber and Osborn (1969) or the discontinuous SDS-polyacrylamide gel method of Laemmli (1970). After electrophoresis the gels were stained for protein with Coomassie Brilliant Blue or frozen and sliced into 2 mm slices which were counted for radioactivity. The gel slices were transferred to scintillation vials and 500 µl of 30% hydrogen peroxide added. The vials were tightly sealed and placed in a 55°C oven overnight. After cooling to room temperature, 10 ml of Aquasol-2 (New England Nuclear) was added and the vials counted to locate the radioactive bands.

Electrophoresis of the total translation products was also performed, by the above methods, on samples both before and after immunoprecipitation.

2.13 Assay of Liver Cyclic AMP

A method similar to those of Gilman (1970) and McManus *et al.* (1972) was used. Rats were killed by exsanguination, and the livers removed and dropped into liquid nitrogen within 10 s of death. Homogenates were prepared from liver in 5 volumes of 5% trichloroacetic acid containing 5 mM theophylline. Insoluble material was removed by centrifugation at 12,100 g for 10 min. Supernatants were extracted

six times with equal volumes of water-saturated diethyl ether, after addition of 0.1 ml of 1 N HCl, to remove trichloroacetic acid. The supernatants were lyophilized and dissolved in appropriate volumes of a buffer containing 50 mM Tris-HCl, pH 7.4 and 4 mM EDTA. Suitable aliquots were assayed for cAMP using the assay kit produced by Amersham Corp, Oakville, Ontario. Controls, in which known amounts of cAMP were added during homogenization, showed that recoveries were not less than 85%. The results have been corrected for losses.

2.14 Assay of Liver Glycogen.

Glycogen was assayed by the procedure of Hassid and Abraham (1956).

2.15 Assay of Serum Glucose.

The method was based on that described by Werner *et al.* (1970). Serum was prepared within 15 min after blood was shed and 0.1 ml volumes were deproteinized by addition of 1.0 ml of 1.6% uranyl acetate in 0.15 M NaCl. Samples were allowed to stand for 15 min, then centrifuged for 2 min in an Eppendorf 3200 centrifuge. The supernatants (0.1 ml) were assayed for glucose using the GOD-Perid glucose assay kit supplied by Boehringer-Mannheim GmbH.

2.16 Assay of Serum Insulin and Cortisol.

Serum was prepared as described under assay of serum glucose. Cortisol was assayed using the RIANEN cortisol ^{125}I-radioimmune assay kit supplied by New England Nuclear. Insulin was measured using the Insulin RIA kit (code IM 78) from Amersham Corporation. Results were expressed in microunits of insulin per milliliter using human insulin as the standard. Thanks go to J.C. Jamieson for his help in performing the hormonal analysis in this thesis.

2.17 Analysis of other Lipogenic Enzymes and Lipid Levels during Adaptive Synthesis and Inflammation.

Rats were starved for 48 h and refed a fat-free diet for 0, 12 and 24 h with inflammation induced, in a similar group, by a 0 h injection of oil of turpentine. Rats were killed at the times indicated, their livers excised, weighed and frozen in liquid N_2.

2.17.1 Lipid analysis.

Lipids were extracted with methanol:chloroform as described by Kates (1972). Frozen liver (6-7 g) was thawed, in 3 ml of water, at room temperature and the tissue ground in a Potter-Elvehjem glass homogenizer. This mixture was extracted with 30 ml of methanol: chloroform (2:1 v/v), centrifuged, the supernatant decanted and the residue re-extracted with 38 ml of methanol:chloroform:water (2:1:0.8 v/v). After centrifugation the supernatants were combined, diluted with 20 ml each of chloroform and water and the phases separated by centrifugation. The lower phase was withdrawn and concentrated in a rotary evaporator at 30-35°C (one ml of benzene was added to remove water traces). The residue was dissolved in 5 ml of chloroform: methanol (2:1 v/v) and stored at -20°C.

The total lipid weight was determined by drying a volume of lipid in a pre-weighed vial.

Triglyceride analysis was performed by a commercial laboratory (we thank John Dick for his assistance) using the enzymatic method of McGraw *et al.* (1979).

Cholesterol was determined by the Liebermann-Burchard reaction as described by Stadtman (1957).

2.17.2 Enzymatic Analysis

Enzymatic analysis was by a modification of the procedure of Diamant *et al.* (1972). A portion of the frozen liver was thawed in 3 volumes of 0.04 M potassium phosphate, pH 7.2, containing 0.1 M sucrose, 0.5 M KCl, 0.03 M EDTA and 0.01 M DTT, and homogenized in a Potter-Elvehjem glass homogenizer at 4°C. The homogenate was centrifuged twice at 10 K for 15 min and the supernatants collected. The combined supernatants were centrifuged at 105,000 g for 60 min in a 60 Ti rotor. The resulting supernatnat was assayed for ATP-citrate lyase and acetyl-CoA carboxylase activity.

2.17.2.1 ATP-citrate lyase assay

The activity of ATP-citrate lyase was determined by the method of Takeda *et al.* (1969) as modified by Lin and Srere (1979). The assay involves coupling with malate-dehydrogenase and measuring the rate of NADH oxidation at room temperature. The reaction mixture contained in 1.0 ml; 100 µmol Tris-HCl pH 8.7, 20 µmol potassium citrate, 10 µmol DTT, 10 µmol $MgCl_2$, 0.5 units malate dehydrogenase, 0.33 µmol CoA, 0.14 µmol NADH and 5 µmol ATP. Because the assay was conducted on crude supernatant fractions a blank without ATP and CoA was subtracted from each value, to account for endogenous NADH oxidation. The enzyme activity was expressed as moles of NADH oxidized/min.

2.17.2.2 Acetyl-CoA carboxylase assay

The activity of Acetyl-CoA carboxylase was determined by the conversion of [^{14}C]-bicarbonate into the carboxyl group of malonyl-CoA as described by Inoue and Lowenstein (1975) and Das (1980). The enzyme was preincubated for 30 min at 37°C in a medium containing 2 mg/ml liver supernatant protein, 60 mM Tris-HCl pH 7.0, 0.1 mM EDTA, 8 mM

$MgCl_2$, 0.3 mM DTT, 10 mM sodium citrate and 0.6 mg/ml fat-free bovine serum albumin.

To assay the enzyme, 100 µl of the preincubated enzyme solution was added to a 1.0 ml assay solution containing 60 mM Tris-HCl pH 7.0, 0.1 mM EDTA, 8 mM $MgCl_2$, 3 mM DTT, 15 mM sodium citrate, 0.6 mg/ml fat-free bovine serum albumin, 5 mM ATP, 0.2 mM acetyl-CoA and 20 mM $NaH^{14}CO_3$ (25 µCi/µM). The reaction was run for 7 min at 37°C and terminated by the addition of 50 µl of 6 N HCl. The reaction mixture was transferred to a scintillation vial and taken to dryness under a gentle stream of air to expel unreacted $^{14}CO_2$. The residue was dissolved in water and counted. The blank was without acetyl-CoA and ATP. Enzyme activity is expressed as nanomoles of malonyl-CoA produced per min.

RESULTS

RESULTS

This thesis is an attempt to more fully elucidate the cellular mechanisms controlling the adaptive synthesis of the lipogenic enzymes in general, and fatty acid synthetase in particular; and to provide some insight into the mechanism of the effects of experimental inflammation on this adaptive synthesis of FAS. The results presented are divided into six parts covering three general areas of investigation. The first section covers the preliminary experiments which provided the framework for the other two areas. The second section, comprised of 3 parts, reports studies of the mechanisms leading to the observed variations in FAS levels. The last section, made up of two parts, includes observations on the corresponding variations in hormonal levels and other aspects of lipogenesis.

1. PRELIMINARY EXPERIMENTS

1.1 Purification of Rat Liver Fatty Acid Synthetase.

Rat liver fatty acid synthetase was purified by the procedures of Burton *et al.* (1965) and Stoops *et al.* (1979) as described in the Experimental Procedures. The specific activity is expressed as either nanomoles of palmitic acid produced per minute per milligram protein or nanomoles of NADPH oxidized per minute per milligram of protein.

To obtain purified enzyme, rats were starved for 48 h and refed a fat-free diet for 48 h prior to killing. The purified enzyme preparation typically yielded 20 to 30 mg of protein, from 30 to 40 g of fresh liver, and consistently had a specific activity of approximately 65 nanomoles of palmitic acid produced (or 910 nanomoles of NADPH oxidized) per minute per milligram of protein. The enzyme was stored at -20°C in 0.5 M potassium phosphate buffer, pH 7.0 containing 1 mM EDTA and 5 mM DTT at a high protein concentration (20-25 mg/ml).

Kinetic analysis of the purified enzyme showed a close similarity between rat liver FAS and the pigeon liver enzyme as previously reported by Katiyar *et al.* (1975). Sedimentation analysis indicated the enzyme sedimented essentially as a single component in 0.5 M potassium phosphate buffer and yielded an approximate molecular weight of 5.4×10^5. Sucrose density gradient centrifugation of the DEAE-cellulose purified enzyme yielded a single distinct protein peak which contained the enzyme activity.

1.2 Preparation and Characterization of the Antiserum to Rat Liver FAS.

Rabbit antiserum to rat liver FAS was induced by injection of either the enzyme from the sucrose density gradient peak or the DEAE-

Figure 9: Quantitative precipitin reaction of rat liver fatty acid synthetase.

The DEAE-cellulose purified enzyme (O——O) or the 105,000 g supernatant (Δ——Δ) was prepared from rats refed a fat-free diet for 48 H, following 48 H starvation. To 220 µg of rabbit anti-FAS antiserum (partially purified by a 0-40 % ammonium sulfate fractionation of rabbit serum) were added the amounts of enzyme indicated. After incubation and centrifugation (as described in Experimental Procedures) the supernatant solutions were assayed for FAS activity.

Enzyme units are given in nanomoles of NADPH oxidized per minute.

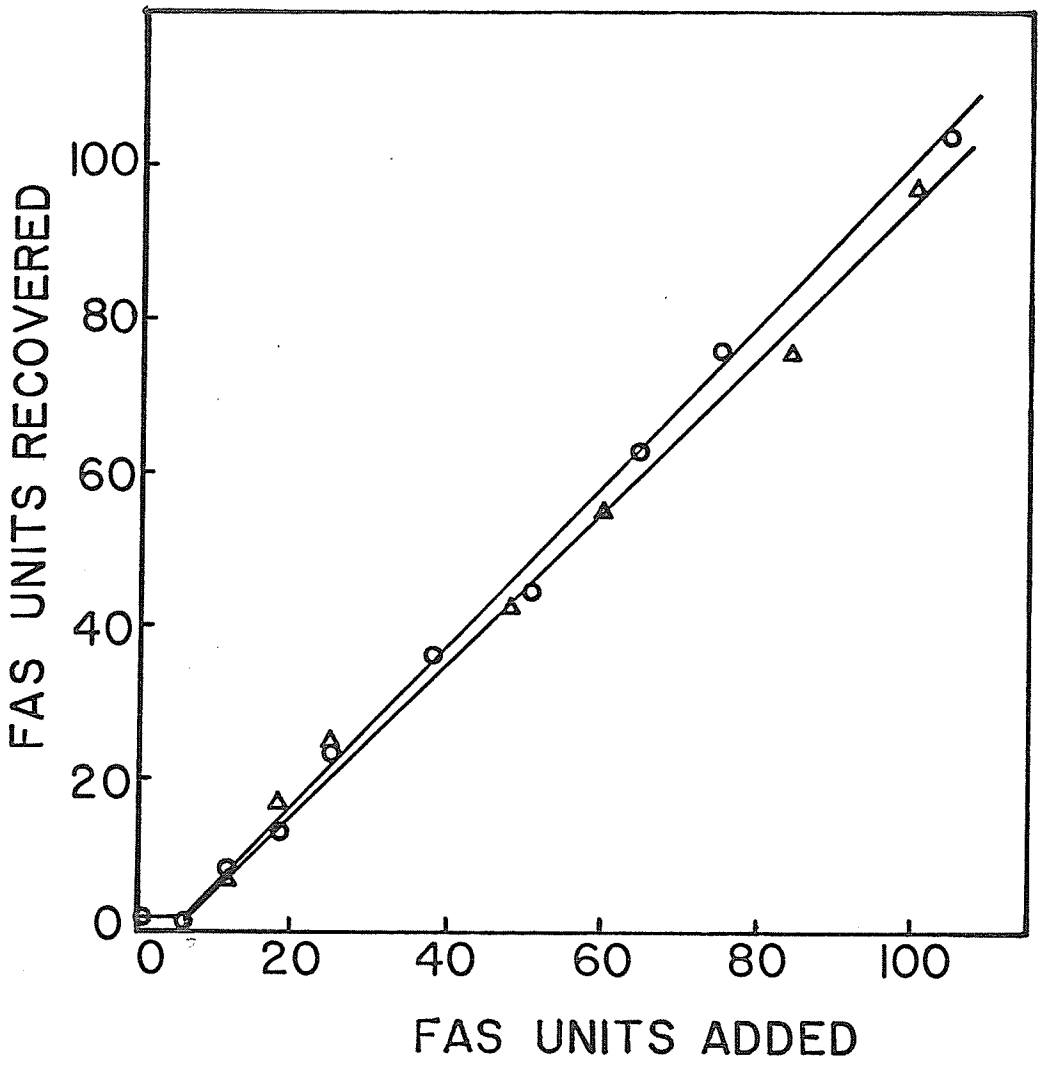
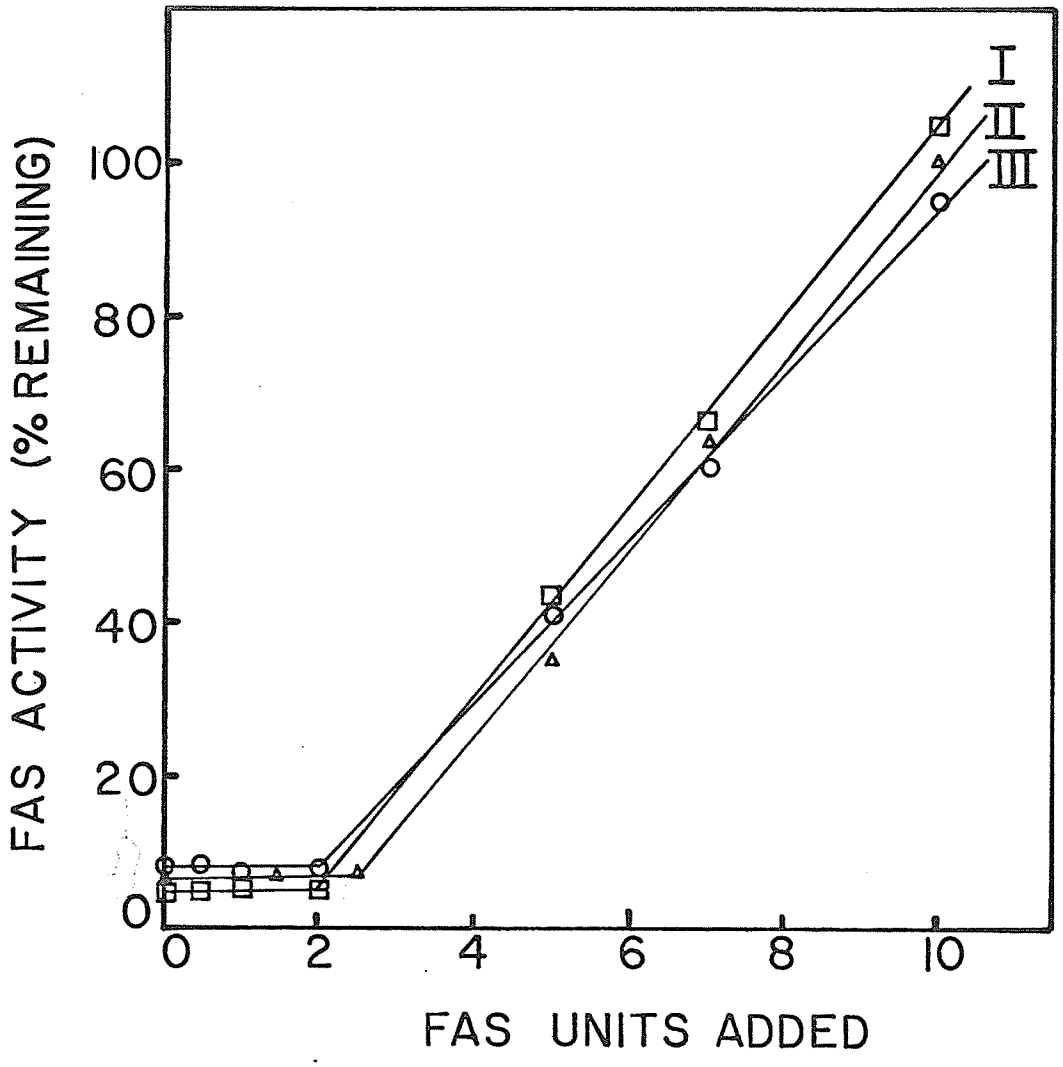

FAS UNITS RECOVERED
0
20
40
60
80
100
0
20
40
60
80
100
FAS UNITS ADDED

Figure 10: Quantitative precipitin reaction of rat liver fatty acid synthetase.

The 105,000 g crude supernatant from three sources, I (□—□) starved 48 H and refed a fat-free diet for 48 H, II (△—△) starved 48 H and III (○—○) starved for 24 H, was added to 50 µg of rabbit anti-FAS antiserum and treated as described in Experimental Procedures.

Enzyme units are nanomoles of NADPH oxidized per minute per mg protein.


FAS ACTIVITY (% REMAINING)
100
80
60
40
20
0
0
2
4
6
8
10
FAS UNITS ADDED
I
II
III

cellulose purified enzyme. Analysis by Ouchterlony double diffusion with both preparation of antisera showed only a single precipitin line with no visible spurring. This indicated the antisera produced were monospecific for rat liver fatty acid synthetase. These single precipitin bands were observed whether the sample tested was purified FAS or the 105,000 g (crude) supernatant.

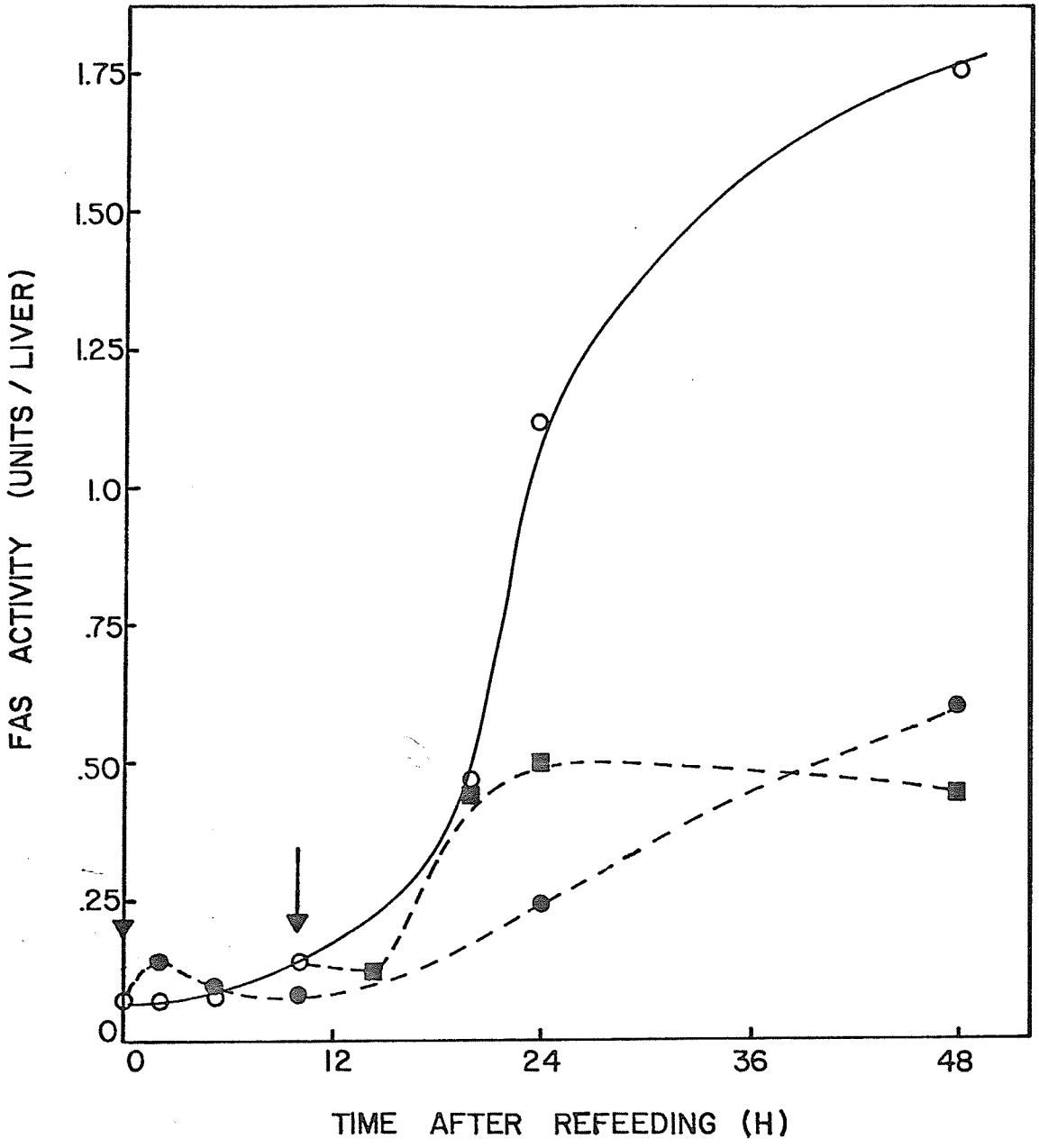
The quantitative precipitin reactions between partially purified anti-FAS antisera and various preparations of enzyme were performed as described by Kabat and Meyer (1961). Figure 9 illustrates the reaction between 220 µg of antiserum and both purified and 105,000 g crude supernatant preparations. Both the purified and the crude preparations were obtained from rats starved for 48 h and refed a fat-free diet for 48 h. The immunochemical equivalence points for both preparations were identical (approximately 33 µg of antiserum per unit of enzyme activity) while the specific activity of the two preparations differed by a factor of 16. The specific activity of the crude enzyme was 49 while that of the DEAE-cellulose purified enzyme was 840 nanomoles of NADPH oxidized per minute per mg of protein. These data indicate the FAS molecules in both the purified and crude preparations had identical catalytic abilities. A number of other equivalence point titrations, with purified and crude preparations, were performed (data not shown) with varying amounts of enzyme and antiserum. All yielded identical results; 30-40 µg of antiserum was required to precipitate one unit of FAS activity (nanomoles of NADPH oxidized per min per mg protein).

Figure 10 illustrates the quantitative precipitin reactions for 50 µg of anti-FAS antiserum and three crude preparations of rat liver FAS: I. starved for 48 h and refed a fat-free diet for 48 h, II.

Figure 11. Suppression of the adaptive synthesis of FAS by experimental inflammation.

Rats were starved for 48 H and then refed a fat-free diet for up to 48 H. Turpentine injections were administered to two different sets of animals at 0 or 10 H after the start of refeeding (indicated by the arrows). Enzyme activity was measured in the 105,000 g supernatant as described in Experimental Procedures.

Enzyme units are micromoles of palmitic acid produced per minute. Each point represents an average value using at least two rats. Fatty acid synthetase activity in non-inflamed rats (O—O), in rats inflamed at 10 H (■--■), and in rats inflamed at 0 H (●--●).


1.75
1.50
1.25
1.0
.75
.50
.25
0
FAS ACTIVITY (UNITS / LIVER)
0
12
24
36
48
TIME AFTER REFEEDING (H)

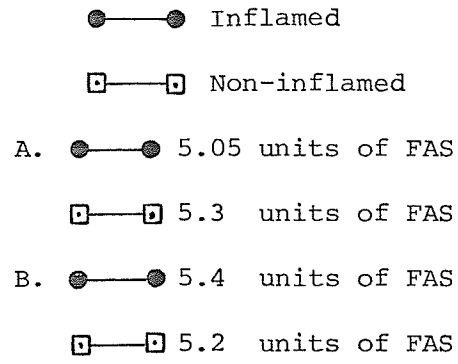
starved for 48 h and III. starved for 24 h prior to killing. The specific activities of the three preparations were 70, 7.5 and 12 nanomoles of NADPH oxidized per minute per mg protein, respectively. All yielded approximately the same equivalence point indicating the enzyme from these preparations had identical catalytic abilities. Results for rats maintained on a normal diet or starved for 48 h and refed a fat-free diet for 96 h were also identical to the three preparations shown in Figure 10.

1.3 The Adaptive Synthesis of Rat Liver FAS and the Effect of Inflammation.

Figure 11 shows the phenomenon of adaptive synthesis of FAS and the influence of inflammation on this response. Rats were starved for 48 h and refed a fat-free diet for up to 48 h. Inflammation was induced at 0 h or 10 h after the start of refeeding. The activity of the hepatic FAS was measured in the 105,000 g supernatant and expressed as micromoles of palmitic acid produced per minute per liver. The levels of FAS rose approximately 25-fold with 48 h refeeding following starvation. This adaptive synthesis was inhibited by the induction of the inflammatory response and the pattern of this inhibition appears dependent upon the time intervals between the start of refeeding and the induction of inflammation and between the induction of inflammation and the time of sacrifice. These results are in agreement with those of Shutler *et al.* (1977).

Figure 12: Immunochemical titration of FAS preparations.

A constant amount of 105,000 g liver supernatant was titrated with anti-FAS serum as described in Experimental Procedures. Animals were starved for 48 H and refed a fat-free diet for 48 H (A) and 20 H (B). Inflammation was induced 10 H after the start of refeeding.



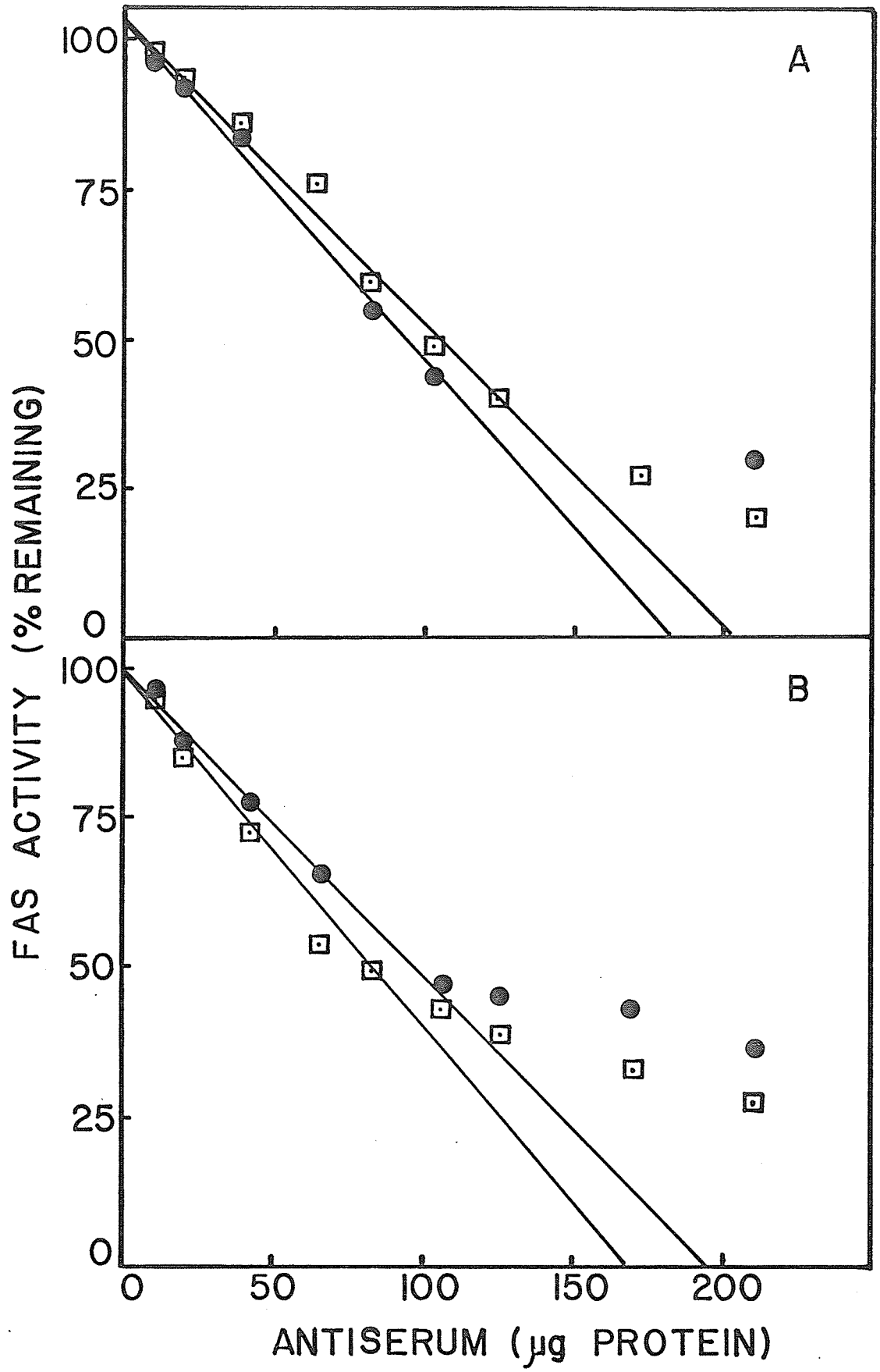
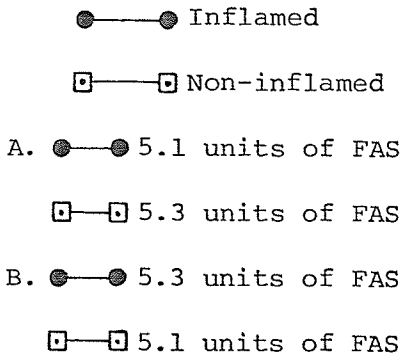

A
B
100
75
50
25
0
FAS ACTIVITY (% REMAINING)
0
50
100
150
200
ANTISERUM (μg PROTEIN)

Figure 13: Immunochemical titration of FAS preparations.

A constant amount of 105,000 g liver supernatant was titrated with anti-FAS serum as described in Experimental Procedures. Animals were starved for 48 H and refed a fat-free diet for 24 H (A) and 15 H (B). Inflammation was induced 5 H after the start of refeeding.



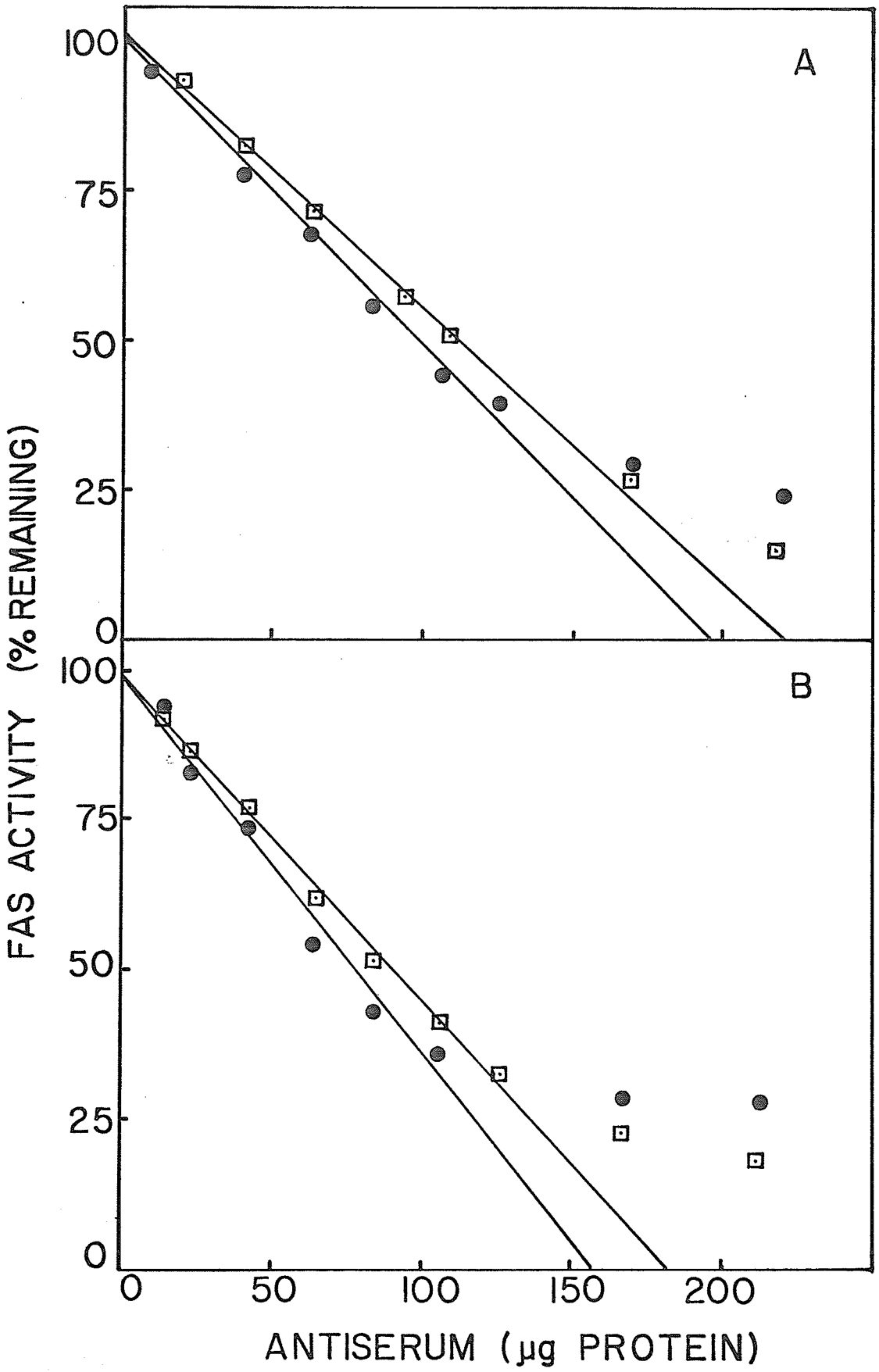
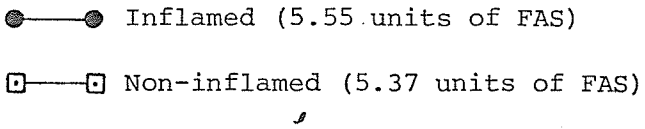
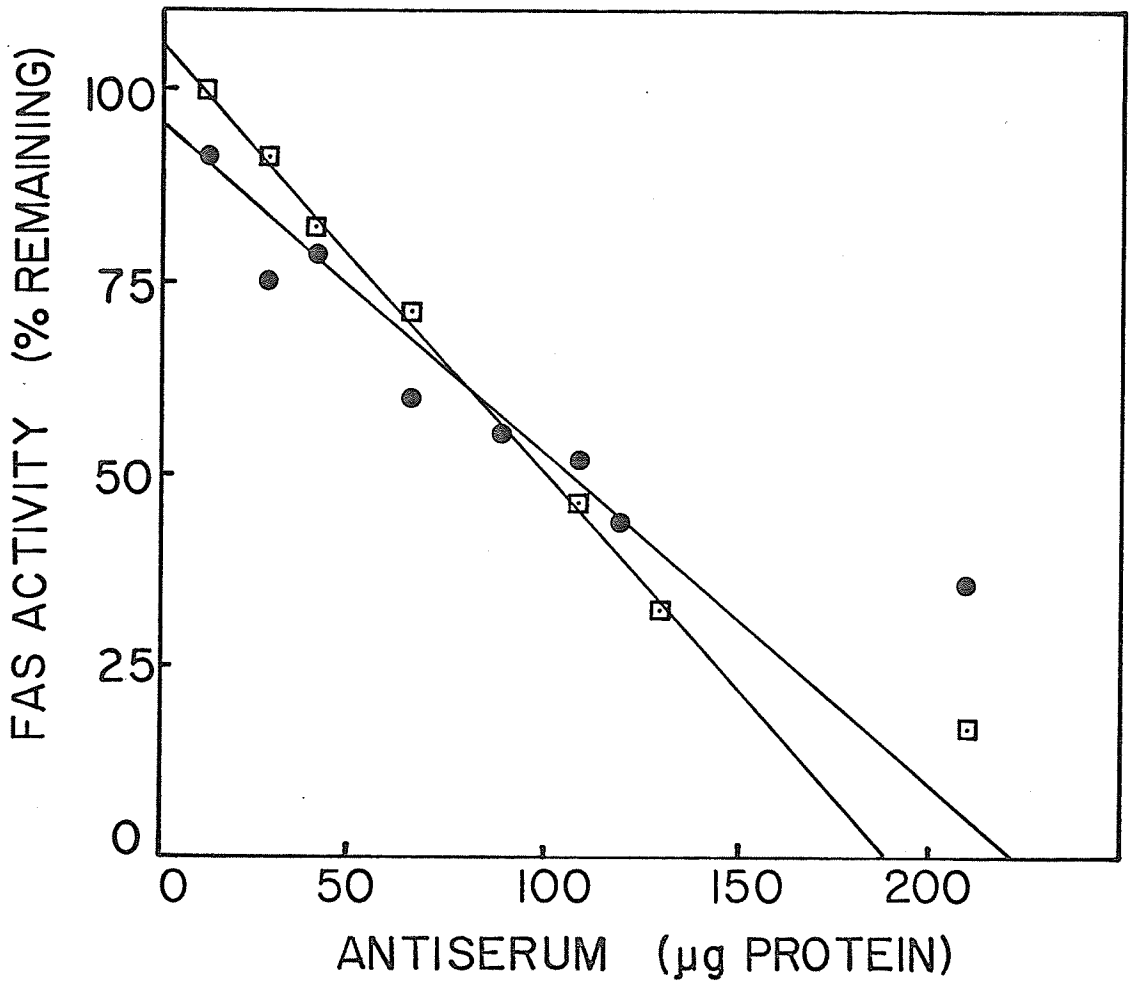

A
B
100
75
50
25
0
FAS ACTIVITY (% REMAINING)
0
50
100
150
200
ANTISERUM (µg PROTEIN)

Figure 14: Immunochemical titration of FAS preparations.

A constant amount of 105,000 g liver supernatant was titrated with anti-FAS serum as described in Experimental Procedures. Animals were starved for 48 H and refed a fat-free diet for 48 H. Inflammation was induced 2.5 H after the start of refeeding.




FAS ACTIVITY (% REMAINING)
100
75
50
25
0
0
50
100
150
200
ANTISERUM (µg PROTEIN)

2. EFFECTS OF EXPERIMENTAL INFLAMMATION ON THE HEPATIC CONCENTRATION OF FAS.

Experimental evidence indicated that the adaptive synthesis of FAS that occurred with refeeding was due to an increase in enzyme content not catalytic ability (Hicks *et al.*, 1965; Burton *et al.*, 1969). As indicated in the previous section and in Craig *et al.* (1972) and Volpe *et al.* (1973) the equivalence point remains constant during the rapid rise in enzyme levels. To investigate whether the suppression of the adaptive synthesis of FAS by inflammation involves similar variations in enzyme content and not catalytic changes, quantitative precipitin reactions were performed to determine the immunochemical equivalence points.

2.1 Immunochemical Equivalence Point Titrations.

Figures 12, 13 and 14 show the results of experiments with inflammation induced 10, 5.0 and 2.5 hours after the start of refeeding. Crude FAS preparations from inflamed and non-inflamed animals, with widely differing specific activities, were treated as described in experimental procedures.

Table 6 summarizes the results of all three experiments and shows no significant difference in the amount of rabbit anti-FAS antiserum required to precipitate 1 unit of FAS activity from any of the crude preparations. This suggests that the FAS molecules from the inflamed and non-inflamed sources had identical catalytic activities and that the observed variations are due to changes in enzyme content.

2.2 Determination of Amounts of FAS Protein by Radial Immunodiffusion.

The quantitative precipitin analysis illustrated that variations in FAS activity occurred while the enzyme equivalence points remained

Table 6. Equivalence Point Determinations of FAS Preparations.

Time of inflammation (Hr after Refeeding)	Time of sacrifice (Hr after refeeding)	FAS specific activity[a]		Equivalence point[b]	
		Control	Inflamed	Control	Inflamed
10	20	20	12	32.7	34.8
10	48	45	13	38.7	35.6
5	15	13	5.5	36.1	31.0
5	24	41	15	42.0	39.0
2.5	48	70	38	35.8	39.5
	Average values			37.1	36.2

[a]Specific activity of FAS is given as nmoles of NADPH oxidized per minute per mg of protein.

[b]Equivalence point is the number of μg of anti-FAS serum required to precipitate 1 unit of FAS activity. Values were calculated from the data shown in Figures 12,13, and 14.

the same. To determine if a correlation exists between the variations in FAS activity and amounts of material cross-reacting with anti-FAS serum, the single radial immunodiffusion method of Mancini *et al.* (1964) as modified by Burton *et al.* (1979) was used.

Preliminary studies with purified enzyme preparations indicated the diameter of the precipitin ring bore a linear relationship to the logarithm of the amount of FAS used over a range of 1-10 µg of purified enzyme. This analysis is similar to that of Fahey and McKelvey (1965) who demonstrated the same relationship with serum immunoglobulins. Mancini *et al.* (1964; 1965) found a linear relationship between the precipitin area and antigen concentration, while Lou and Shanbrom (1967) related antigen concentration to the diameter of the precipitin ring. Rasanen (1974) pointed out that the discrepancies in analysis may be due to some of the factors varying in the different systems; molecular weight of the antigen and its heterogeneity, corresponding properties of the antibody, its affinity as well as specificity, the mutual concentrations of antigen and antibody and the medium used. Attempts to treat the experimental data, obtained with FAS, by the above methods resulted in non-linear correlations while the relationship of log of the antigen concentration versus diameter routinely yielded a straight line with a correlation coefficient of 0.995 or better.

Standards of purified FAS were run, on each plate, simultaneously with samples of crude FAS preparations obtained from non-inflamed and inflamed animals. The crude enzyme was prepared as described and for each sample a number of dilutions were used for comparative purposes. Photographs of the R.I.D. plates were made and measurements of the average diameter of each precipitin ring were performed.

Figure 15: Determination of FAS levels by radial immunodiffusion.

Rats were starved for 48 H and refed a fat-free diet for various lengths of time as indicated. Inflammation was induced 10 H after the start of refeeding. After killing at the appropriate times, the material precipitated by anti-FAS serum was measured by radial immunodiffusion as described in Experimental Procedures. Each well contained 5 µl of 105,000 g supernatant (except for those containing purified FAS) from rats treated as detailed below:

A: 1-5)
F: 5-9) Purified FAS standards 1.17, 2.33, 5.85, 7.75 and 11.7 µg, respectively.

H: 8*,9; B:7*,8; E: 6 refed fat-free diet for 48 H
H: 4*,5,6*,7; B: 3*,4,5*,6; E: 7,8* refed 48 H plus inflamm.

H: 2*,3; B 1*,2; D: 9 refed 24 H
H: 1; G: 1*; C: 1,2* refed 24 H plus inflammation

G: 2,3*; C: 3,4* refed 20 H
G:4,5*; C: 5,6* refed 20 H plus inflammation

E: 2,3*; B:9*; A: 8 refed 15 H
A: 6,7*; E: 4,5* refed 15 H plus inflammation

G: 6,7*,8*; C: 7,8*; F: 4*; D: 1,2* refed 10 H
F: 2,3*; D: 3,4*,8 refed 2.5 H
F: 1; E: 1*; D: 5,6*,7 starved 48 H, no refeeding

* indicates a 1:1 dilution of the 105,000 g supernatant was used.

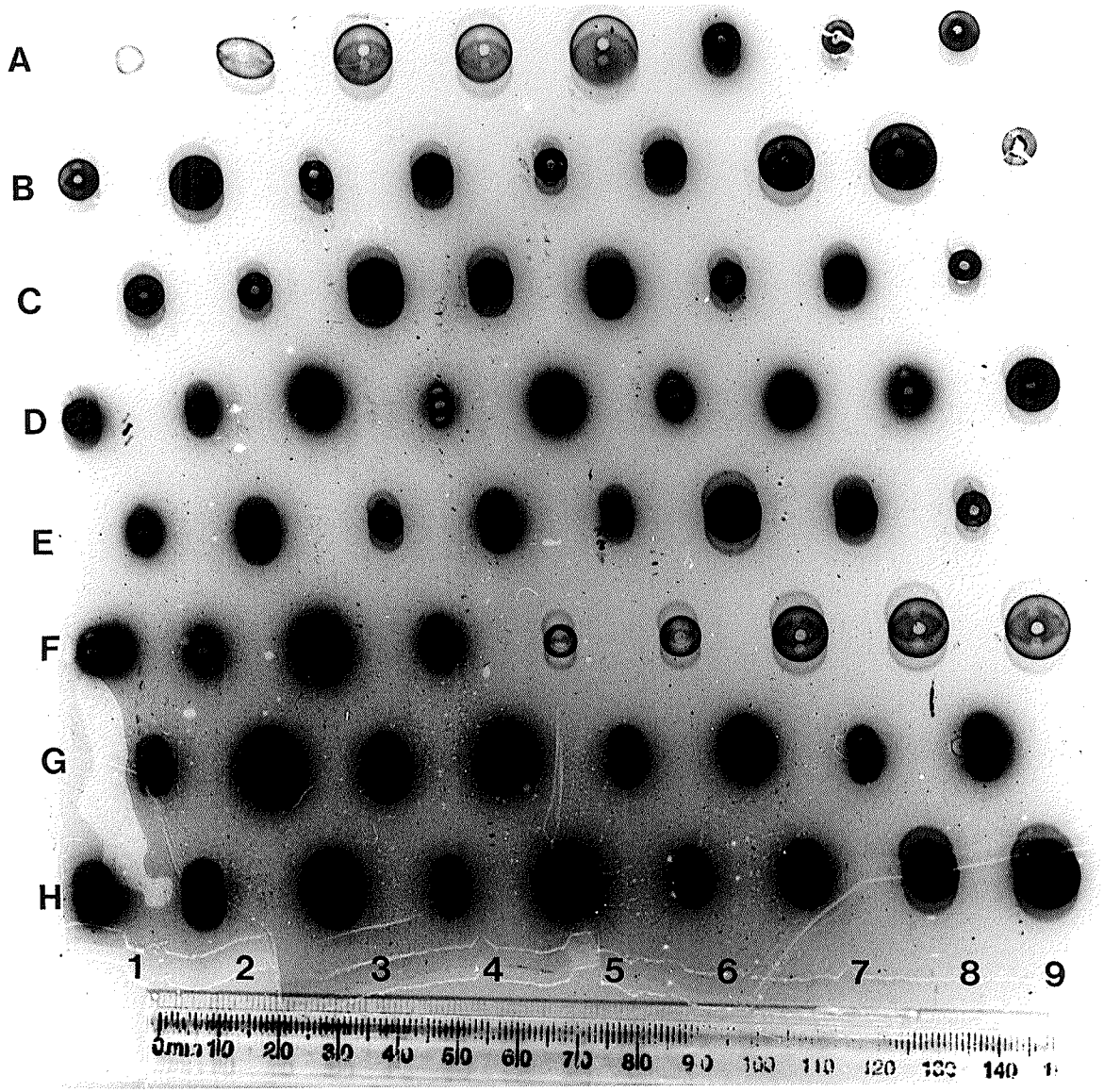

A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
8
9

Figure 16: Standard plot of amount of purified FAS versus precipitin ring diameter.

Radial immunodiffusion was carried out as described in the Experimental Procedures using purified FAS. The ring diameters were taken from Figure 15.

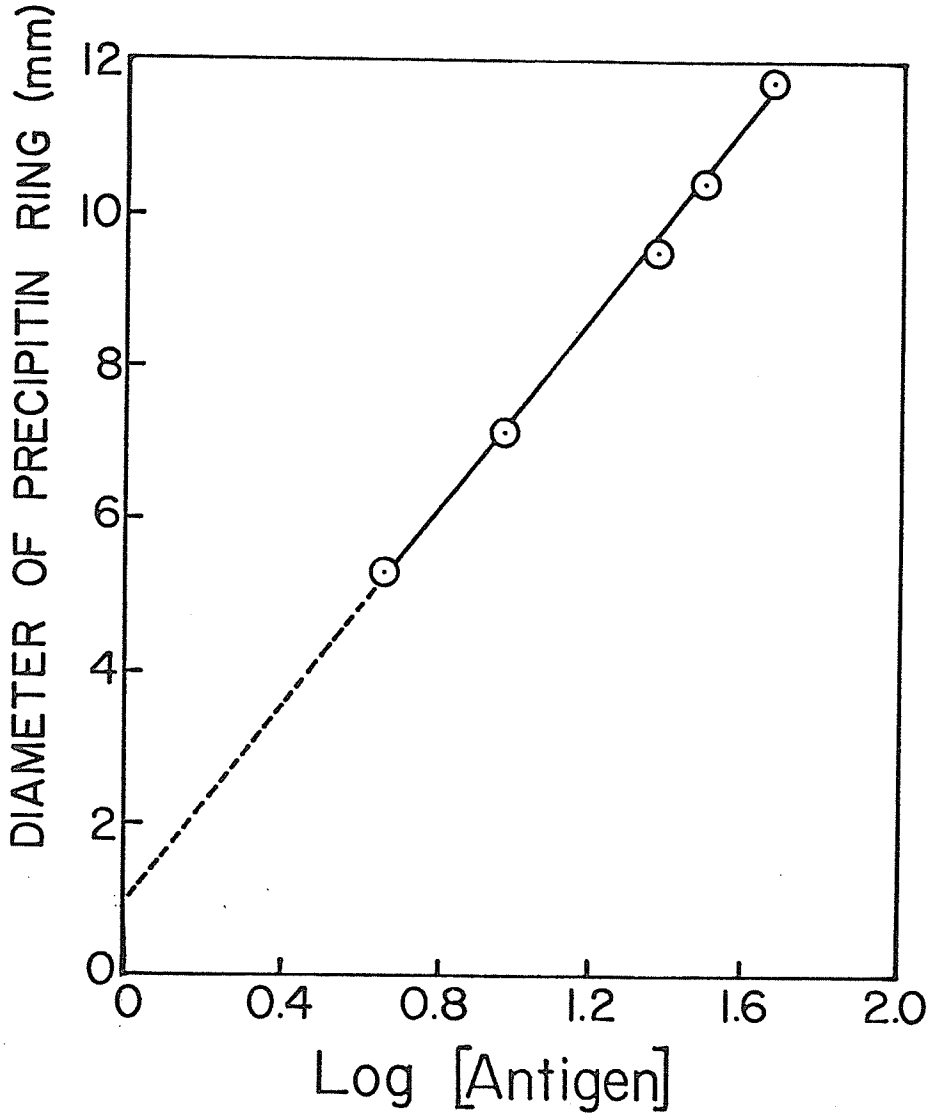
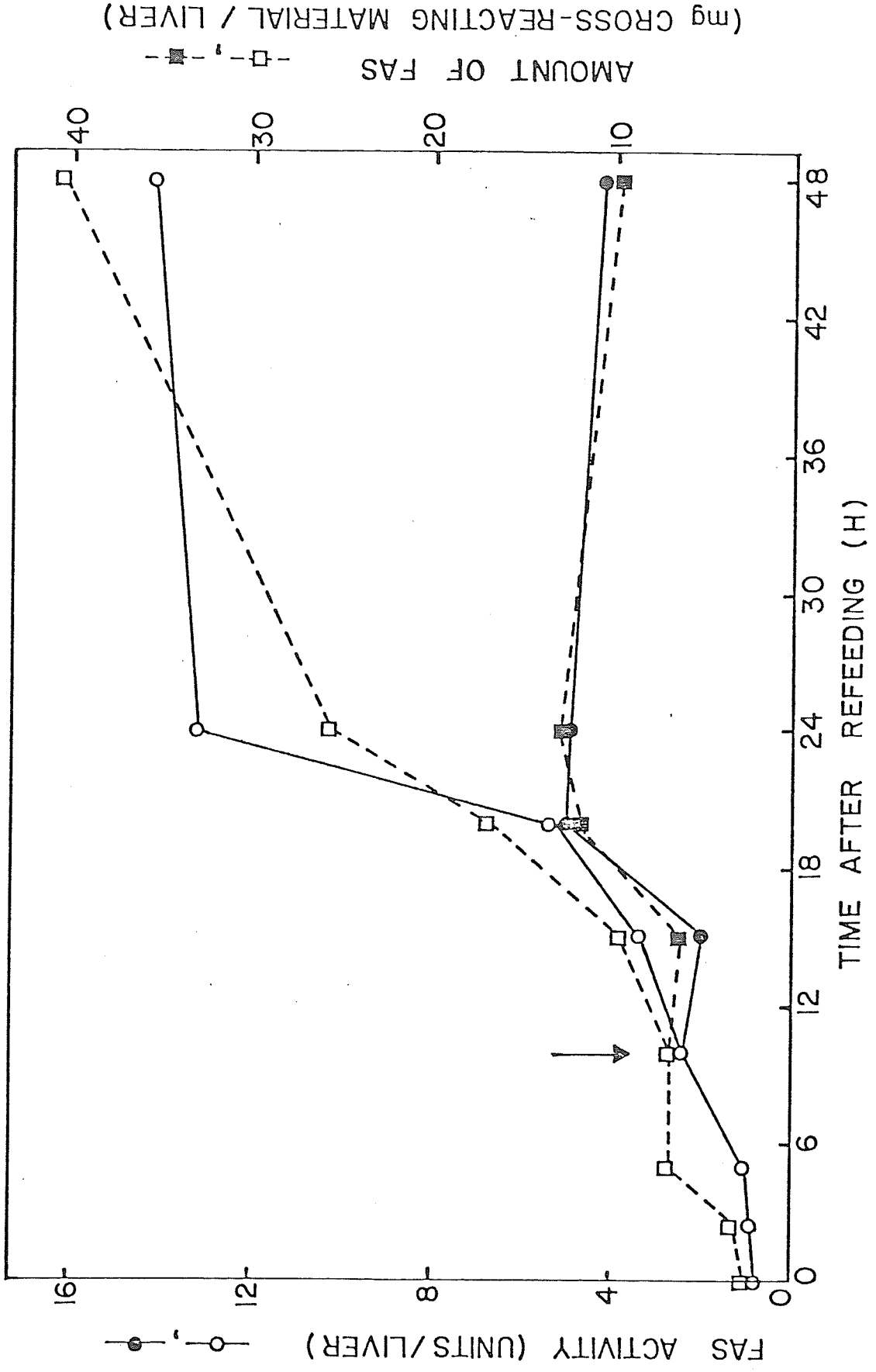

DIAMETER OF PRECIPITIN RING (mm)
12
10
8
6
4
2
0
0
0.4
0.8
1.2
1.6
2.0
Log [Antigen]

Figure 17: Effect of inflammation at 10 H of refeeding on the adaptive synthesis of FAS.

Rats were starved for 48 H and refed a fat-free diet. Inflammation was induced 10 H after the start of refeeding (arrow). After killing at the appropriate times, FAS activity was measured spectrophotometrically, and the material precipitated by anti-FAS serum was measured by radial immunodiffusion. Each point represents an average value obtained using two rats. Data for inflamed animals are indicated by the solid symbols, while the open symbols indicate non-inflamed animals.

Individual values varied from this average by less than $\pm$ 10 %.


AMOUNT OF FAS
(mg CROSS-REACTING MATERIAL / LIVER)
40
30
20
10
FAS ACTIVITY (UNITS/LIVER)
16
12
8
4
0
0
6
12
18
24
30
36
42
48
TIME AFTER REFEEDING (H)

2.2.1 Inflammation experimentally-induced 10 h after the start of refeeding.

Figures 15 and 16 illustrate the radial immunodiffusion (R.I.D.) plate and the standard curve, respectively, for an experiment where inflammation was induced 10 h after the start of refeeding. Rats were starved for 48 h and refed a fat-free diet for up to 48 h. Animals to be inflamed were given a subcutaneous injection of oil of turpentine 10 h after the start of refeeding. The 105,000 g liver supernatants were prepared as described and applied to the wells in the R.I.D. plates.

Figure 17 shows the correlation between the changes in the amounts of material cross-reacting with anti-FAS serum with the changes in FAS activity in both non-inflamed and inflamed animals. After 48 h of refeeding the FAS activity levels increased 25-fold for the non-inflamed and 5-fold for the inflamed animals. The levels of cross-reacting material similarily increased 20-fold and 3-fold. These relationships hold true for the other time intervals of refeeding.

2.2.2 Inflammation experimentally-induced 5.0 h after the start of refeeding.

Figures 18 and 19 illustrate the radial immunodiffusion (R.I.D.) plate and the standard curve, respectively, for an experiment where inflammation was induced 5.0 h after the start of refeeding. Rats were starved for 48 h and refed a fat-free diet for up to 24 h. Levels of cross-reacting material were obtained for both non-inflamed and inflamed animals.

The relationships between changes in the amount of cross-reacting material and the variations in FAS activity are shown in Figure 20. After 24 h of refeeding the enzyme activity levels have increased 14-fold and 6-fold for non-inflamed and inflamed animals, respectively.

Figure 18: Determination of FAS levels by radial immunodiffusion.

Inflammation was induced 5.0 h after the start of refeeding. Other details are given in the legend to Figure 15. Each well contained:

C: 1-5)
E: 6-10) Purified FAS standards 11.7, 7.8, 5.85, 2.35 and 1.17 µg, respectively.

A: 3#,4**,5*,6,7#,8**,9*,10; D: 8* refed fat-free diet
E: 1#,2**,3*,4; F: 1,2*,3**,4#; G: 7*,8** for 24 h.

A: 1,2*; B: 1**,2,3*,4**; D: 9*; E: 5 refed for 24 h plus
F: 6*,7**,8,9*,10**; G: 9*,10 inflammation

C: 6,7*,8**; D:1*,2*,3** refed 20 h
C: 9,10**; D: 4,5**,6**,10** refed 20 h plus inflammation

B: 5,6*,7**,8,9*,10**; D: 7 starved 48 h;
F: 5*; G: 1,2*,3**,4,5*,6** no refeeding

*indicates 1:1 dilution of 105,000 g supernatant was used.

**indicates a 1:5 dilution was used.

# indicates a 1:10 dilution was used.

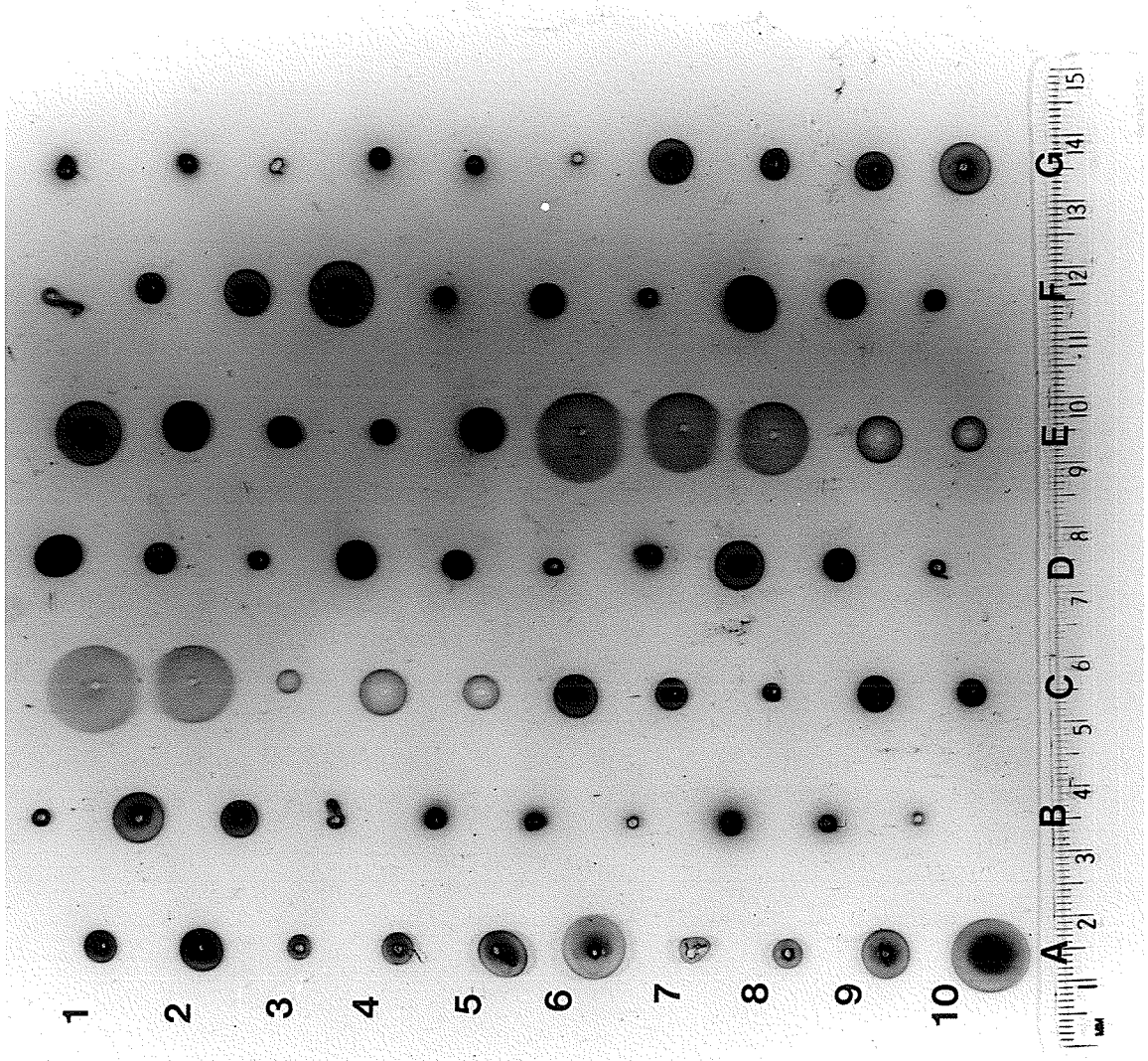

1
2
3
4
5
6
7
8
9
10
A
B
C
D
E
F
G

Figure 19: Standard plot of amount of purified FAS versus precipitin ring diameter.

Radial immunodiffusion was carried out as described in the Experimental Procedures using purified FAS. The ring diameters were taken from Figure 18.

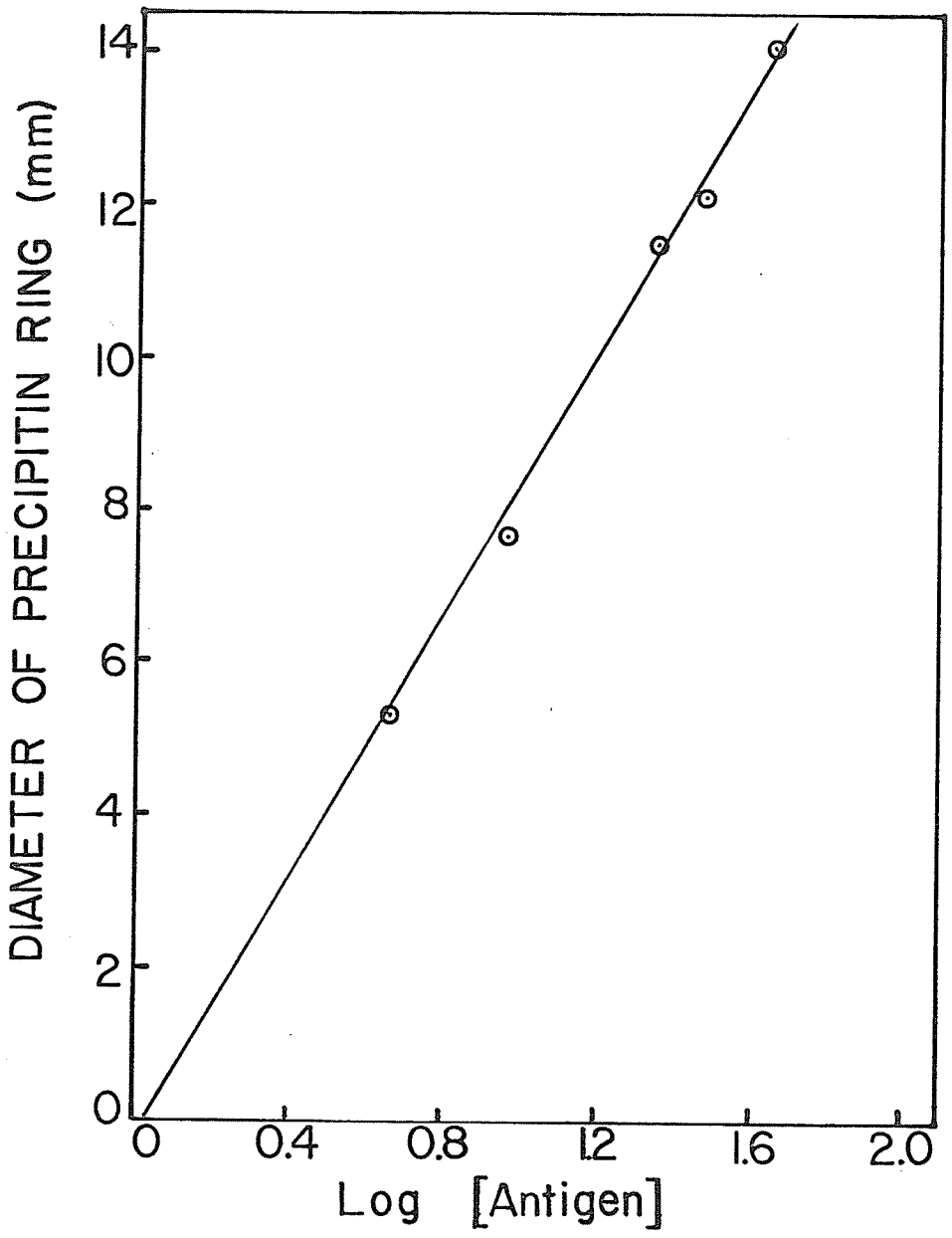
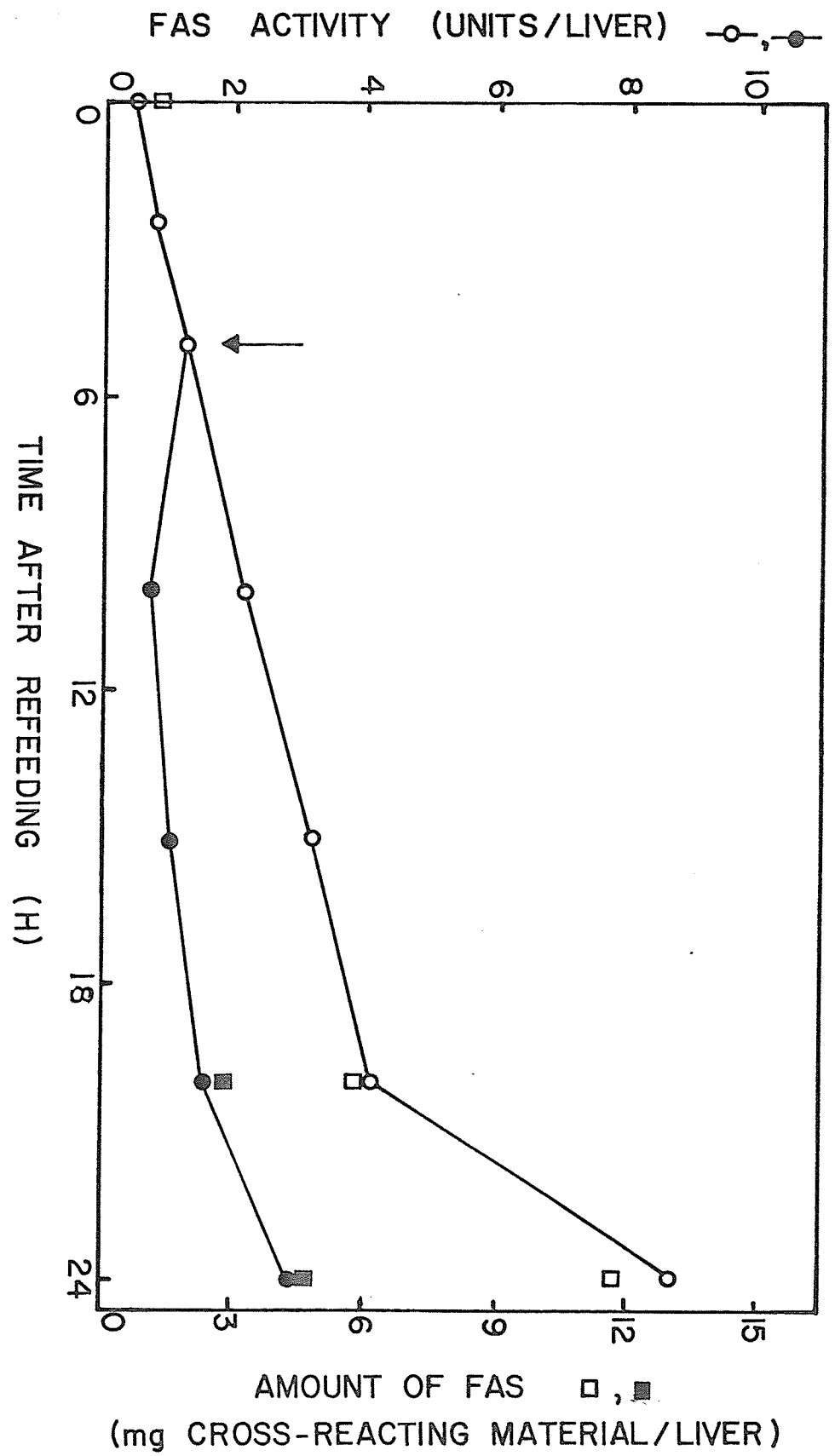

DIAMETER OF PRECIPITIN RING (mm)
0
2
4
6
8
10
12
14
0
0.4
0.8
1.2
1.6
2.0
Log [Antigen]

Figure 20: Effect of inflammation at 5.0 H of refeeding on the adaptive synthesis of FAS.

Rats were starved for 48 H and refed a fat-free diet. Inflammation was induced at 5.0 H after the start of refeeding (arrow). After killing at the appropriate times, FAS activity was measured spectrophotometrically, and the material precipitated by anti-FAS serum was measured by radial immunodiffusion. Each point represents an average value obtained using two rats. Data for inflamed animals are indicated by the solid symbols, while the open symbols indicate non-inflamed animals. Individual values varied from this average by less than $\pm$ 12%.


FAS ACTIVITY (UNITS/LIVER) ○, ●
0
2
4
6
8
10
TIME AFTER REFEEDING (H)
0
6
12
18
24
AMOUNT OF FAS □, ■
(mg CROSS-REACTING MATERIAL/LIVER)
0
3
6
9
12
15

The levels of cross-reacting material increased 10-fold and 3-fold under the same conditions. The levels of FAS increased much slower in this experiment than in the other experiments for reasons which were not discovered. The lower levels of FAS and longer lag phase in the induction were observed both for activity and amounts of cross-reacting material.

2.2.3 Inflammation experimentally-induced 2.5 h after the start of refeeding.

Figures 21 and 22 illustrate the R.I.D. plate and standard curve, respectively, for an experiment where inflammation was induced 2.5 h after the start of refeeding. Figure 22 also illustrates the effect of increasing well diameter and sample volume on the standard curve. The increased well diameter appears to manifest itself by a larger Y intercept while the slope of the line closely parallels that of the normal well size. The R.I.D. plate for the larger well diameter (4 mm) is not shown but the results obtained were similar to those from the 2.5 mm well diameter R.I.D. plate which are presented in Figure 23.

After 48 h of refeeding the enzyme activity levels were increased 30-fold for the non-inflamed and 12-fold for the inflamed animals. The levels of material cross-reacting with anti-FAS serum were increased 35-fold and 5-fold for non-inflamed and inflamed animals under the same conditions.

2.2.4 Inflammation experimentally-induced 1.5 h after the start of refeeding.

The R.I.D. plate and standard curve, for an experiment where inflammation was induced 1.5 h after the start of refeeding, are presented in Figures 24 and 25, respectively. A control R.I.D. plate was also run containing non-specific rabbit antiserum (i.e. had a high

Figure 21: Determination of FAS levels by radial immunodiffusion.

Inflammation was induced 2.5 h after the start of refeeding. Other details are given in the legend to Figure 15. Each well contained:

E: 1-5)
I: 3-7) purified FAS standards 11.7, 7.8, 5.85, 2.35 and 1.17 µg, respectively.
A: 6,7 7.8 and 1.17 µg, respectively.

C: 4,5; G: 6*,7; H: 7,8* refed fat-free diet for 48 h
A: 1*; C: 6: H: 5,6* refed 48 h plus inflammation

C: 7; G: 6*,7; H: 7,8* refed 24 h
A: 2; B: 8; G: 2*,3,4*,5 refed 24 h plus inflammation

B: 7; F: 1*; G: 1 refed 20 h
F: 2,3*; B: 6 refed 20 h plus inflammation

B: 5; F: 4,5* refed 15 h
A: 3; B: 4; F: 6,7* refed 15 h plus inflammation

B: 3: E: 7*; F: 8 refed 9 h
B: 2; D: 8; E: 6* refed 9 h plus inflammation

B: 1; C: 4*,5,6*,7 refed 5 h
A: 4,5: C: 1*; D: 1,2*,3 refed 5 h plus inflammation

C: 2,3* refed 2.5 h

I: 1*,2 starved 48 h, no refeeding

*indicates 1:1 dilution of 105,000 g supernatant was used.

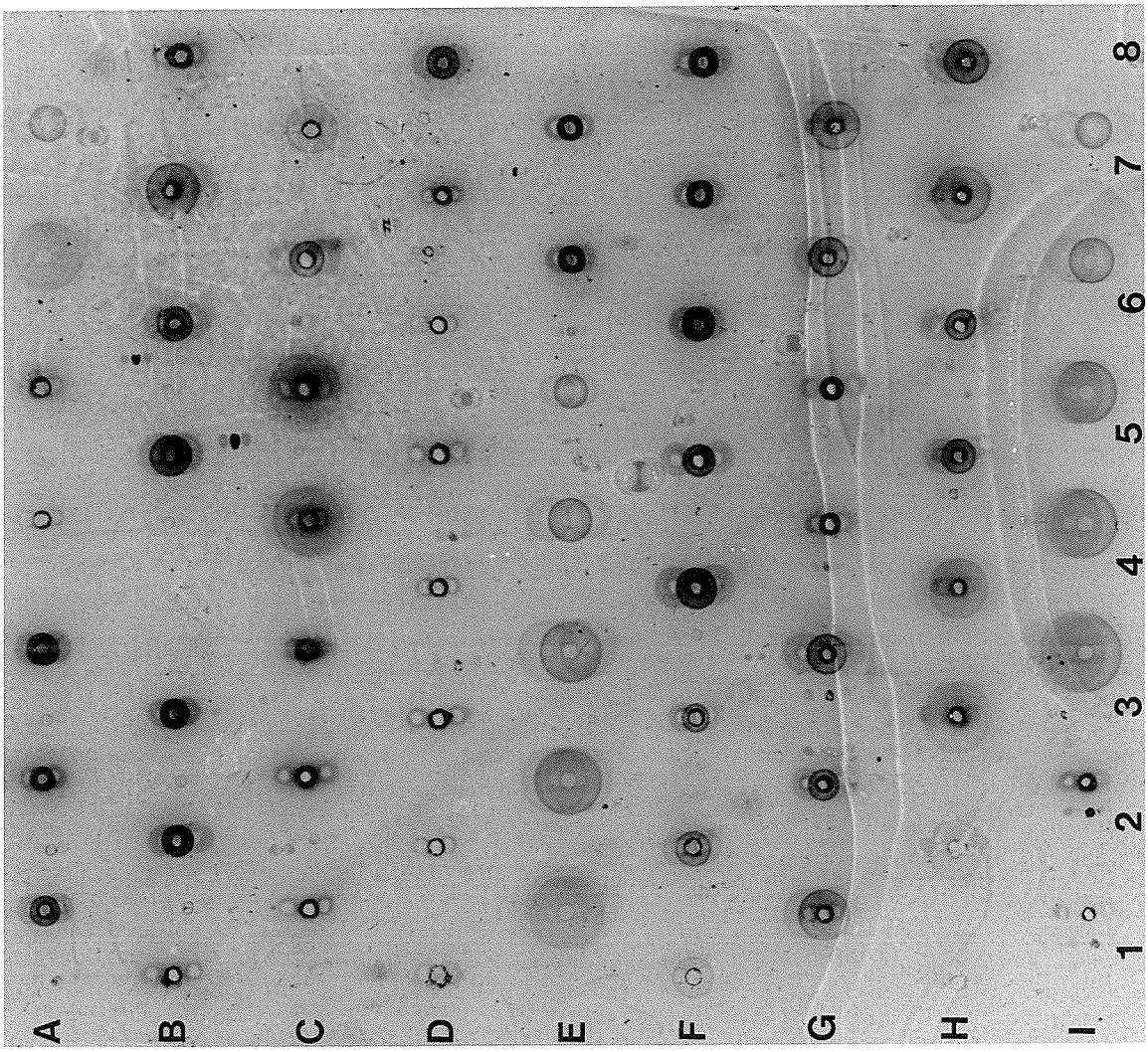

A
B
C
D
E
F
G
H
I
1
2
3
4
5
6
7
8

Figure 22: Standard plot of amount of purified FAS versus precipitin ring diameter.

Radial immunodiffusion was carried out as described in the Experimental Procedures using purified FAS. The ring diameters were taken from Figure 21 (⊙—⊙, for 2.5 mm well diameters); larger well diameters (4 mm) are represented by the ⊡—⊡ slope (R.I.D. plate not shown).

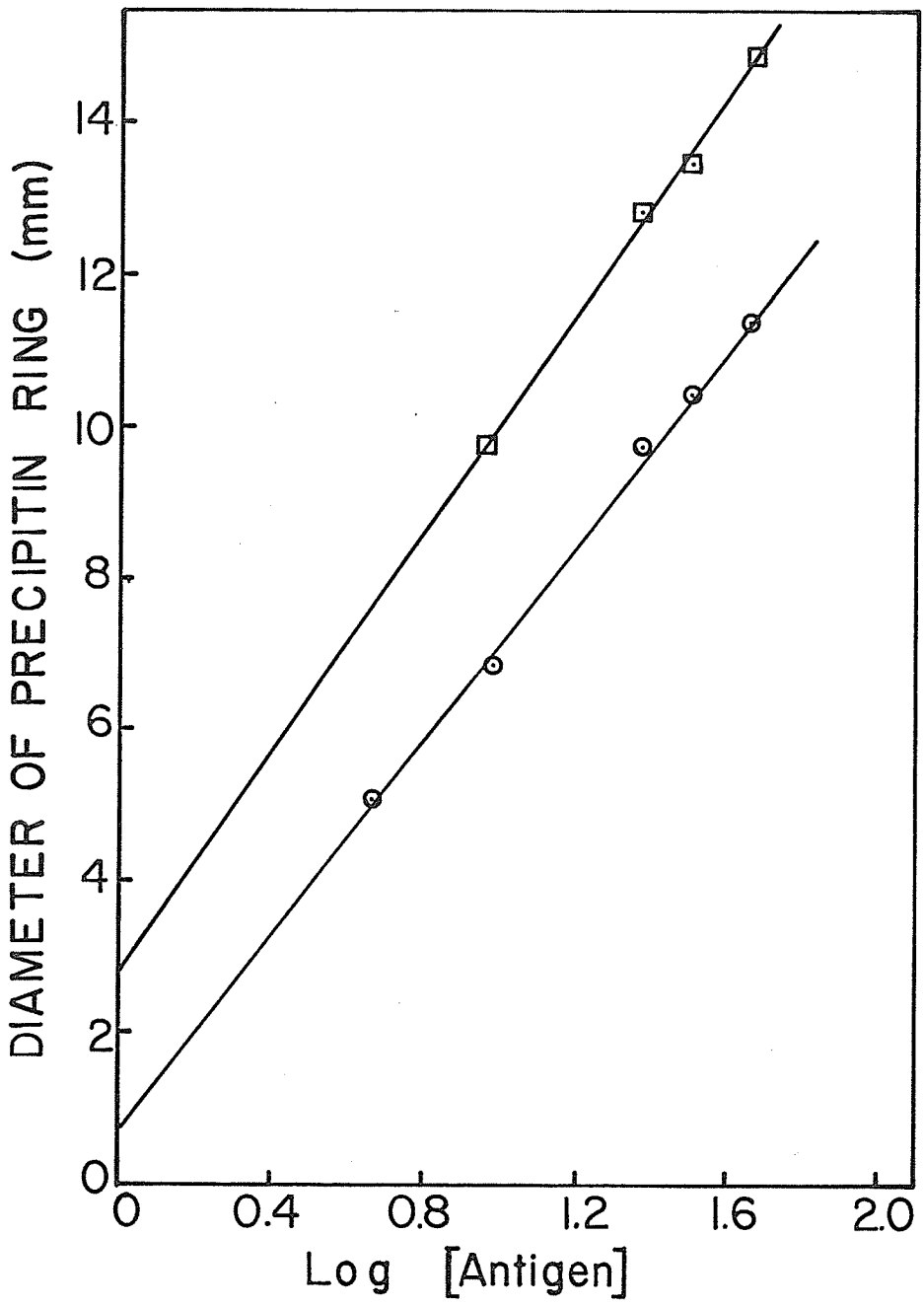

DIAMETER OF PRECIPITIN RING (mm)
0
2
4
6
8
10
12
14
0
0.4
0.8
1.2
1.6
2.0
Log [Antigen]

Figure 23: Effect of inflammation at 2.5 H of refeeding on the adaptive synthesis of FAS.

Rats were starved for 48 H and refed a fat-free diet. Inflammation was induced at 2.5 H after the start of refeeding (arrow). After killing at the appropriate times, FAS activity was measured spectrophotometrically, and the material precipitated by anti-FAS serum was measured by radial immunodiffusion. Each point represents an average value obtained using two rats. Data for inflamed animals are indicated by the solid symbols, while the open symbols indicate non-inflamed animals. Individual values varied from this average by less than $\pm$ 12%.

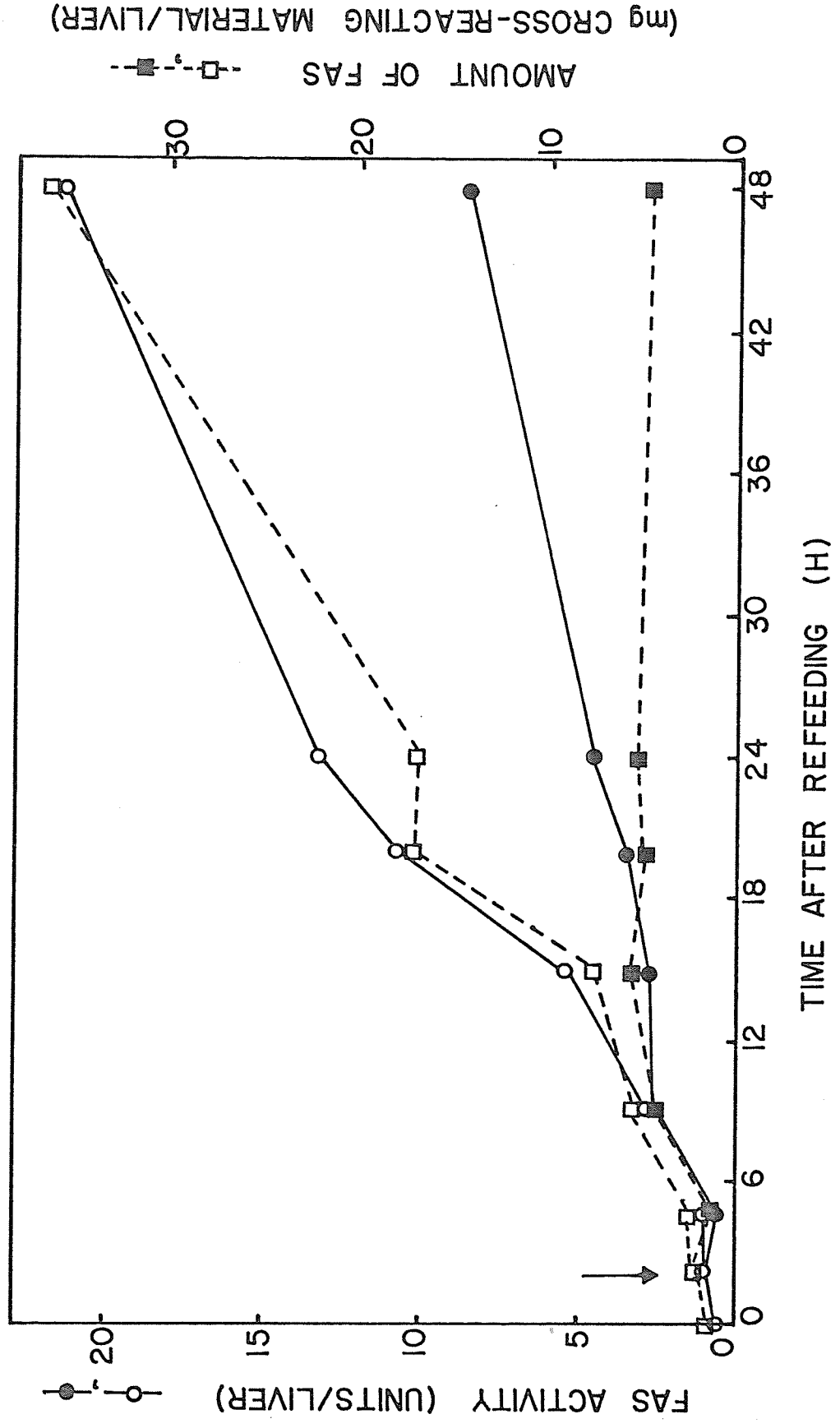

FAS ACTIVITY (UNITS/LIVER)
AMOUNT OF FAS
(mg CROSS-REACTING MATERIAL/LIVER)
TIME AFTER REFEEDING (H)
0
5
10
15
20
30
20
10
0
6
12
18
24
30
36
42
48

Figure 24: Determination of FAS levels by radial immunodiffusion.

Inflammation was induced 1.5 h after the start of refeeding. Other details are given in the legend to Figure 15. Each well contained:

E: 3-7)
I: 1-6) standard purified FAS 1.17, 2.35, 5.85, 7.8 and 11.7 µg, respectively.

A: 1,6*; B: 1,2*,3,4* refed fat-free diet for 48 h
C: 2*,5*; B: 5,7*,8* refed 48 h plus inflammation

A: 3; C: 5*,6,7*; D: 8 refed 24 h
C: 1*,2,3*,4 refed 24 h plus inflammation

D: 1*,2,3*; E: 2 refed 20 h
D: 4,5*,6,7* refed 20 h plus inflammation

F: 2,3*,4,5* refed 15 h
E: 1*; F: 6,7*,8 refed 15 h plus inflammation

A: 4; G: 4*,5,6*,7 refed 9 h
F:1*, G:1,2*,3 refed 9 h plus inflammation

H: 3*,4 refed 5 h
H: 1*,2 refed 5 h plus inflammation

A: 7*; H: 7*,8 refed 2.5 h
H: 5*,6 refed 215 h plus inflammation

I: 6*,7 starved 48 h; no refeeding

*indicates 1:1 dilution of 105,000 g supernatant was used.

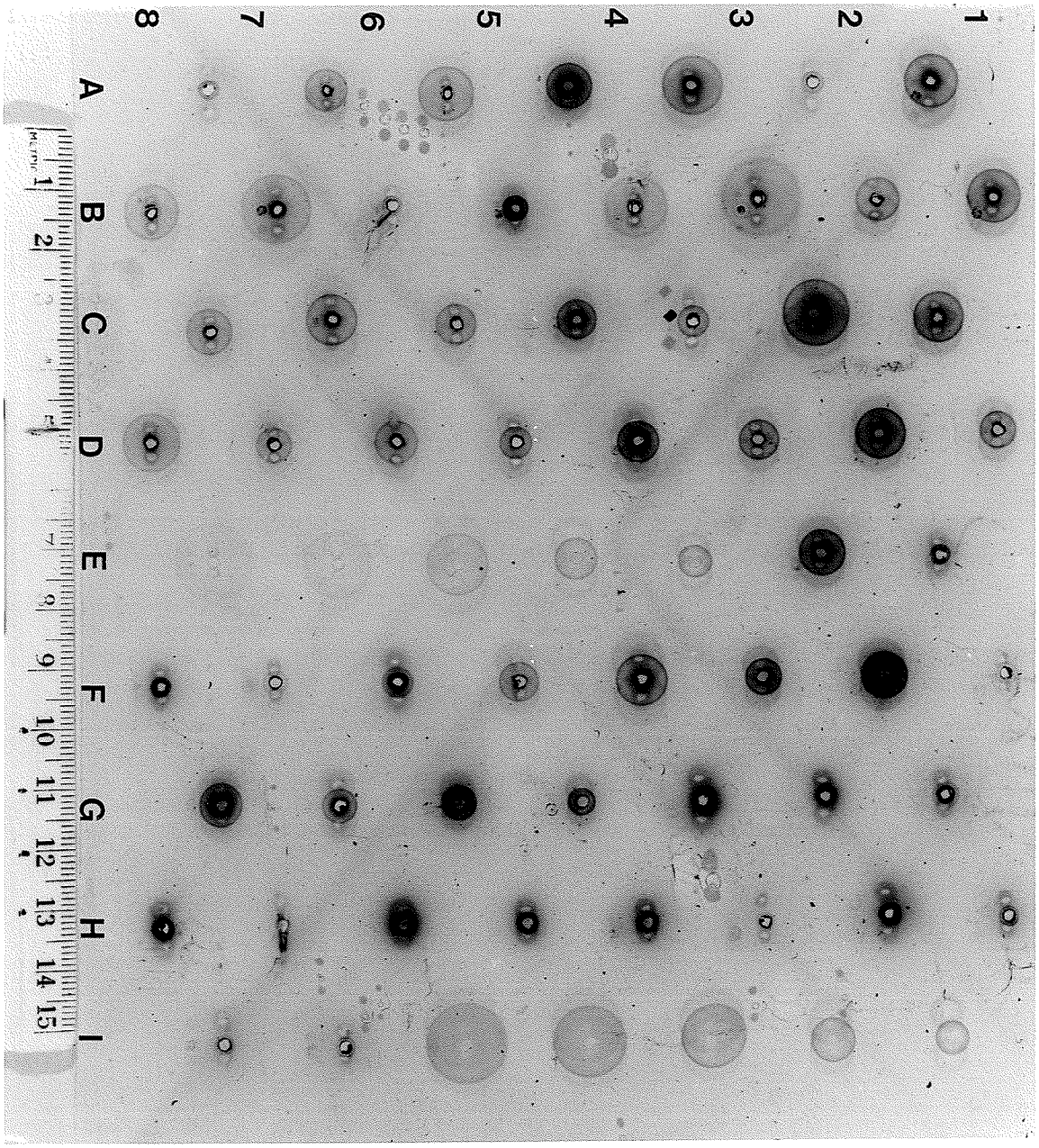

1
2
3
4
5
6
7
8
A
B
C
D
E
F
G
H
I
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 25: Standard plot of amount of purified FAS versus precipitin ring diameter.

Radial immunodiffusion was carried out as described in the Experimental Procedures using purified FAS. The ring diameters were taken from Figure 24.

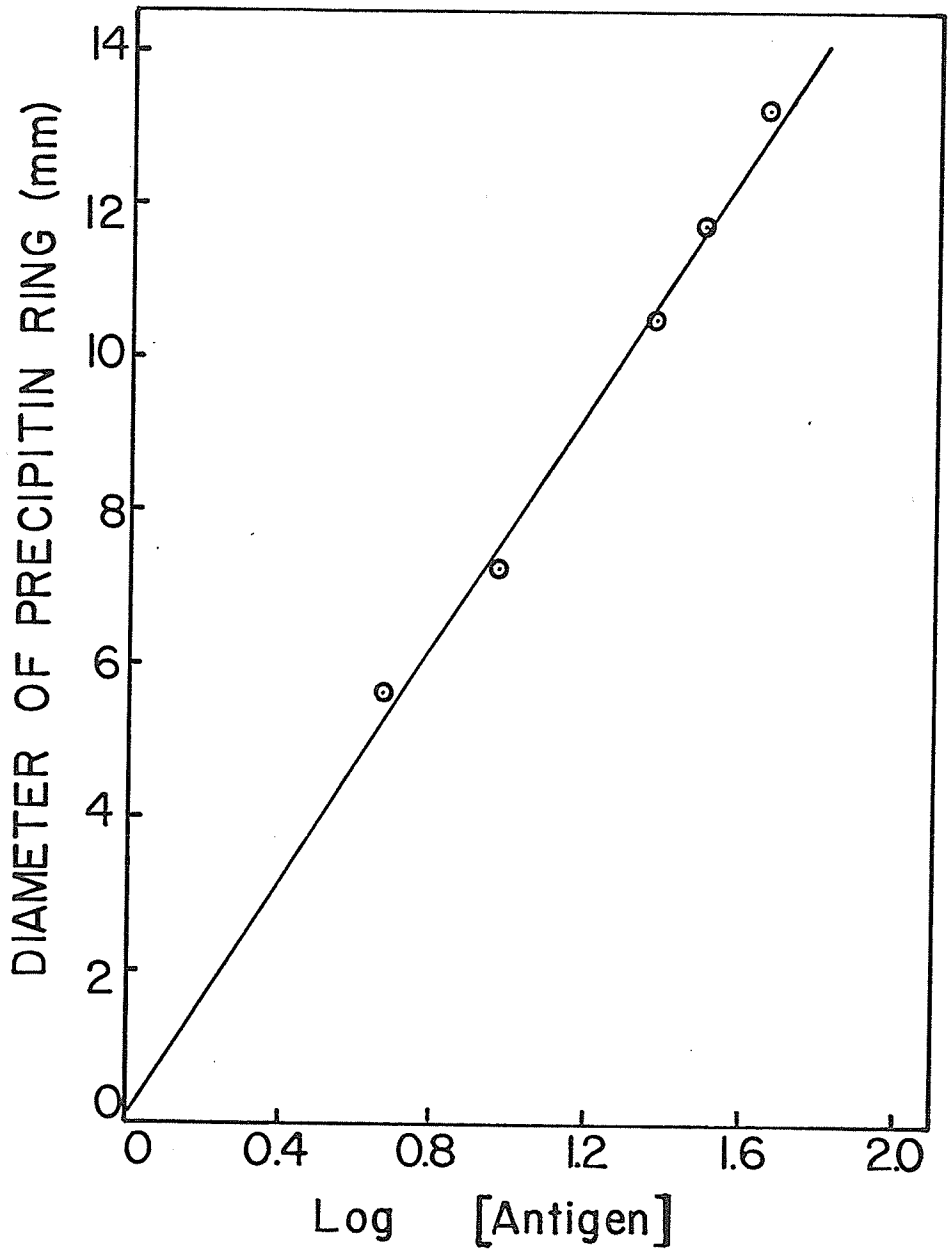
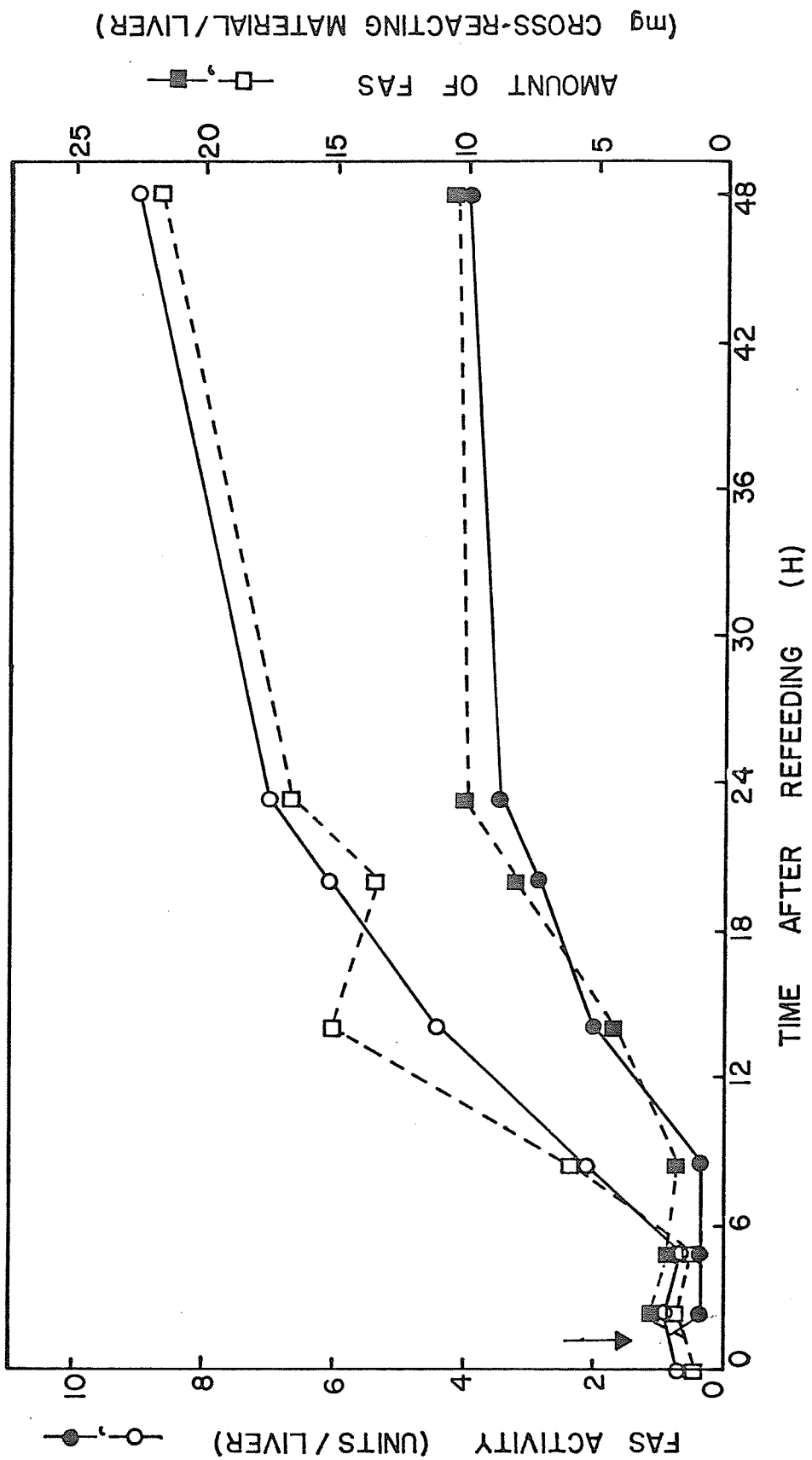

DIAMETER OF PRECIPITIN RING (mm)
14
12
10
8
6
4
2
0
0
0.4
0.8
1.2
1.6
2.0
Log [Antigen]

Figure 26: Effect of inflammation at 1.5 H of refeeding on the adaptive synthesis of FAS.

Rats were starved for 48 H and refed a fat-free diet. Inflammation was induced 1.5 H after the start of refeeding (arrow). After killing at the appropriate times, FAS activity was measured spectrophotometrically, and the material precipitated by anti-FAS serum was measured by radial immunodiffusion. Each point represents an average value obtained using two rats. Data for inflamed animals are indicated by the solid symbols, while the open symbols indicate non-inflamed animals. Individual values varied from this average by less than $\pm$ 12%.


FAS ACTIVITY (UNITS / LIVER)
AMOUNT OF FAS (mg CROSS-REACTING MATERIAL / LIVER)
TIME AFTER REFEEDING (H)
0
2
4
6
8
10
0
5
10
15
20
25
0
6
12
18
24
30
36
42
48

titre of natural antibodies) and showed no precipitin lines developing (data not shown).

After 48 h of refeeding a fat-free diet the non-inflamed animals had a 15-fold increase in activity and a 16-fold increase in cross-reacting material. Similarily the inflamed animals showed a 7-fold increase in activity and an 8-fold increase in cross-reacting material. These relationships are presented in Figure 26.

2.2.5 Inflammation experimentally-induced 0 h after the start of refeeding.

Figures 27 and 28 represent the radial immunodiffusion plates for an experiment where inflammation was induced at the start of refeeding. Figure 27 represents a normal experiment where the agarose medium contained 0.25 M potassium phosphate buffer, pH 7.0, while the plate in Figure 28 also contained 2.5 mM dithiothreitol. The standard curves for both plates are presented in Figure 29. Addition of dithiothreitol appeared to cause a slight increase in precipitin ring diameter perhaps indicating the dithiothreitol acted as a reducing agent interfering with the precipitin reaction. The slopes of the two lines were nearly identical over the range studied.

The relationships between the changes in the amount of cross-reacting material and the variations in FAS activity are shown in Figure 30 (from R.I.D. plate lacking dithiothreitol). After 48 h of refeeding the enzyme activity increased 36-fold for non-inflamed and 13-fold for inflamed animals while the amount of cross-reacting material increased 32-fold and 16-fold, respectively.

Figure 27: Determination of FAS levels by radial immunodiffusion.

Inflammation was induced 0 h after the start of refeeding. Other details are given in the legend to Figure 15. Each well contained:

G: 3-7 standard purified FAS
11.7, 7.8, 5.85, 2.35 and 1.17 µg, respectively.
A: 1,2 7.8 and 5.85 µg,
E: 5,6,7 1.17, 5.85 and 11.7 µg, respectively.

A: 3; C: 1; D: 7; E: 1,2,5,6, refed fat-free diet 48 h
D: 8; E: 3,4 refed 48 h plus inflammation

C: 2; E: 7; F: 1 refed 24 h
C: 3; F: 2,3 refed 24 h plus inflammation

C: 4; F: 4,5 refed 12.5 h
A: 4; B: 1; F: 6,7 refed 12.5 h plus inflammation

A: 5; B: 2; F: 8; G: 1 refed 8 h
B: 3; G: 2 refed 8 h plus inflammation

A: 6; B: 4,6; D: 1,3 refed 4 h
A: 7; B: 5; D: 2 refed 4 h plus inflammation

B: 8; D: 4 refed 2 h
D: 5 refed 2 h plus inflammation

B: 7; D: 6 starved 48 h; no refeeding

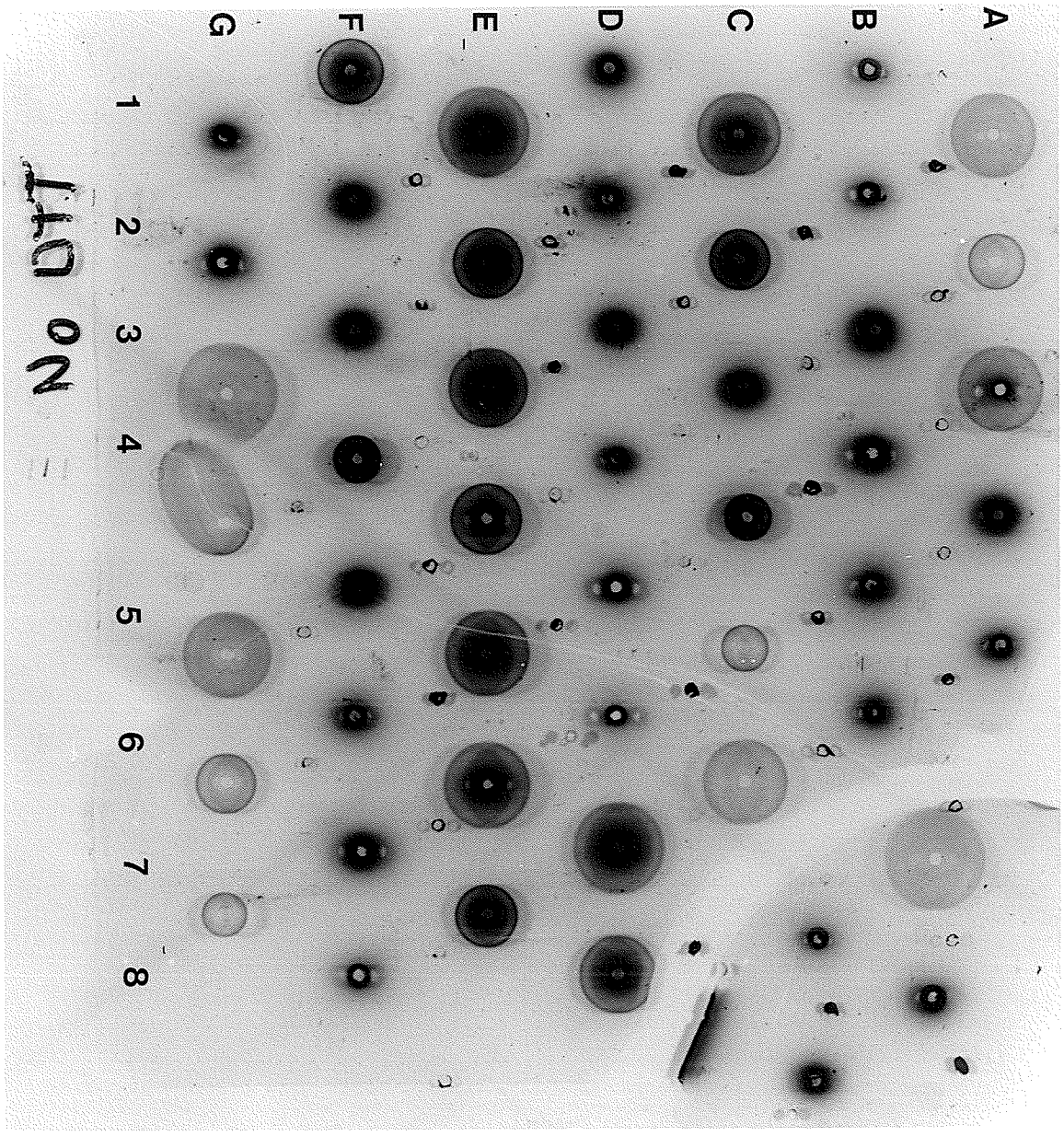

A
B
C
D
E
F
G
1
2
3
4
5
6
7
8
ON
OFF

Figure 28: Determination of FAS levels by radial immunodiffusion.

Inflammation was induced 0 h after the start of refeeding. Other details are given in the legend to Figure 15 except that the gel contained 2.5 mM DTT.

Each well contained:

A: 3-7)
E: 7-3) standard purified FAS 11.7, 7.8, 5.85, 2.35 and 1.17 µg, respectively.

A: 2*; B: 1,2*,3,8*; C: 5,6*,7; G: 3,5 refed fat-free diet for 48 h
B: 4*,5,6*,7; I: 4 refed 48 h plus inflammation

C: 1,2*; D:1,2*; G: 6; I: 5 refed 24 h
C: 3,4*; D: 3,4*; G: 7; I: 6 refed 24 h plus inflammation

D: 5,6,; H: 1; I: 7 refed 12.5 h
D: 7,8; H: 2 refed 12.5 h plus inflammation

E: 1,2; I: 1 refed 8 h
F: 1,2; I: 2 refed 8 h plus inflammation

F: 3,4,7,8; H: 3,5 refed 4 h
F: 5,6; H: 4 refed 4 h plus inflammation

A: 1; H: 8 starved 48 h, no refeeding

*indicates 1:1 dilution of 105,000 g supernatant was used.

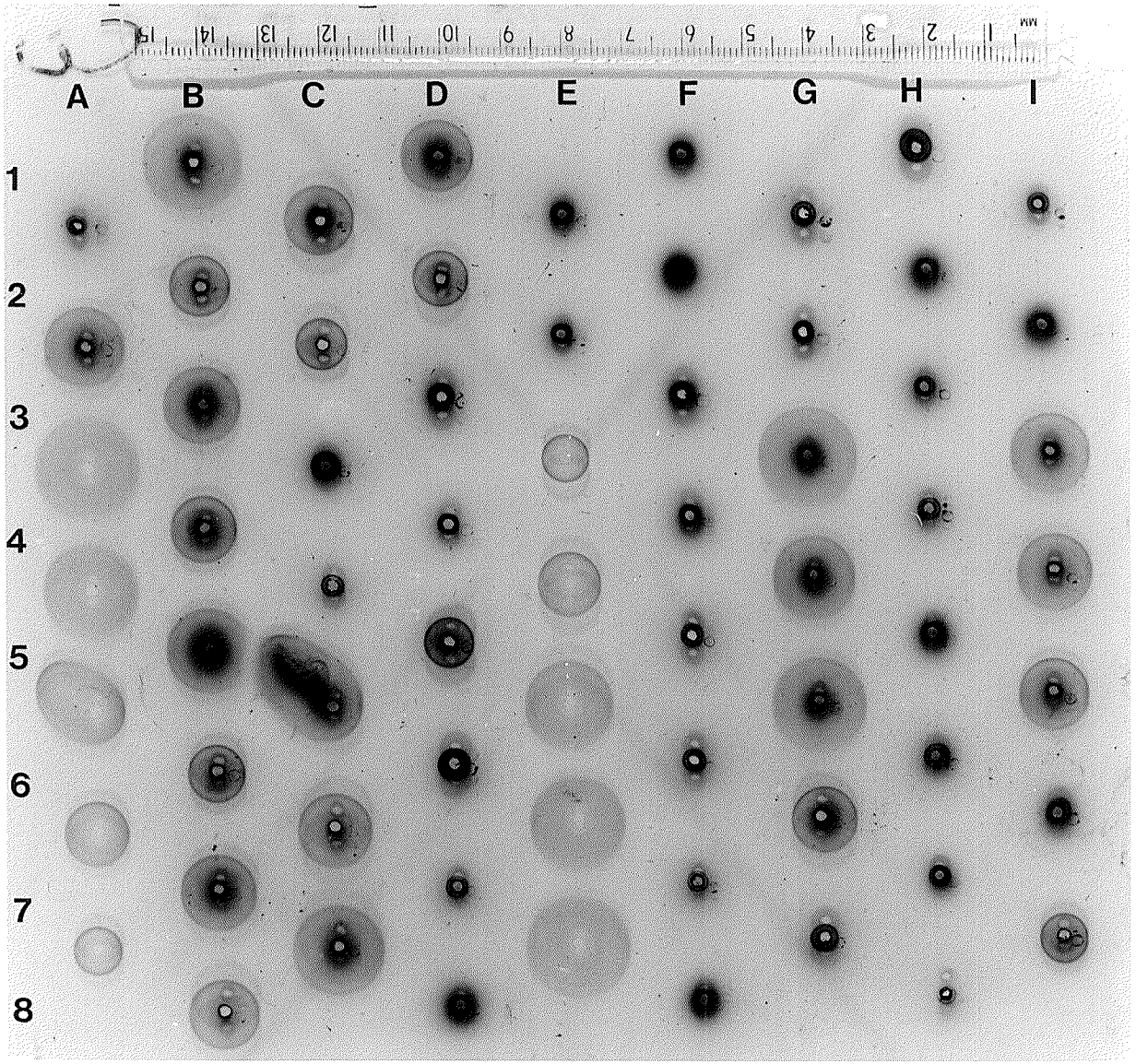

A
B
C
D
E
F
G
H
I
1
2
3
4
5
6
7
8

Figure 29: Standard plots of amounts of purified FAS versus precipitin ring diameter.

Radial immunodiffusion was carried out as described in the Experimental Procedures using purified FAS. The ring diameters were taken from Figure 27 (for R.I.D. plate containing no DTT O—O). The standard from the R.I.D. plate containing 2.5 mM DTT is represented by the □—□ slope.

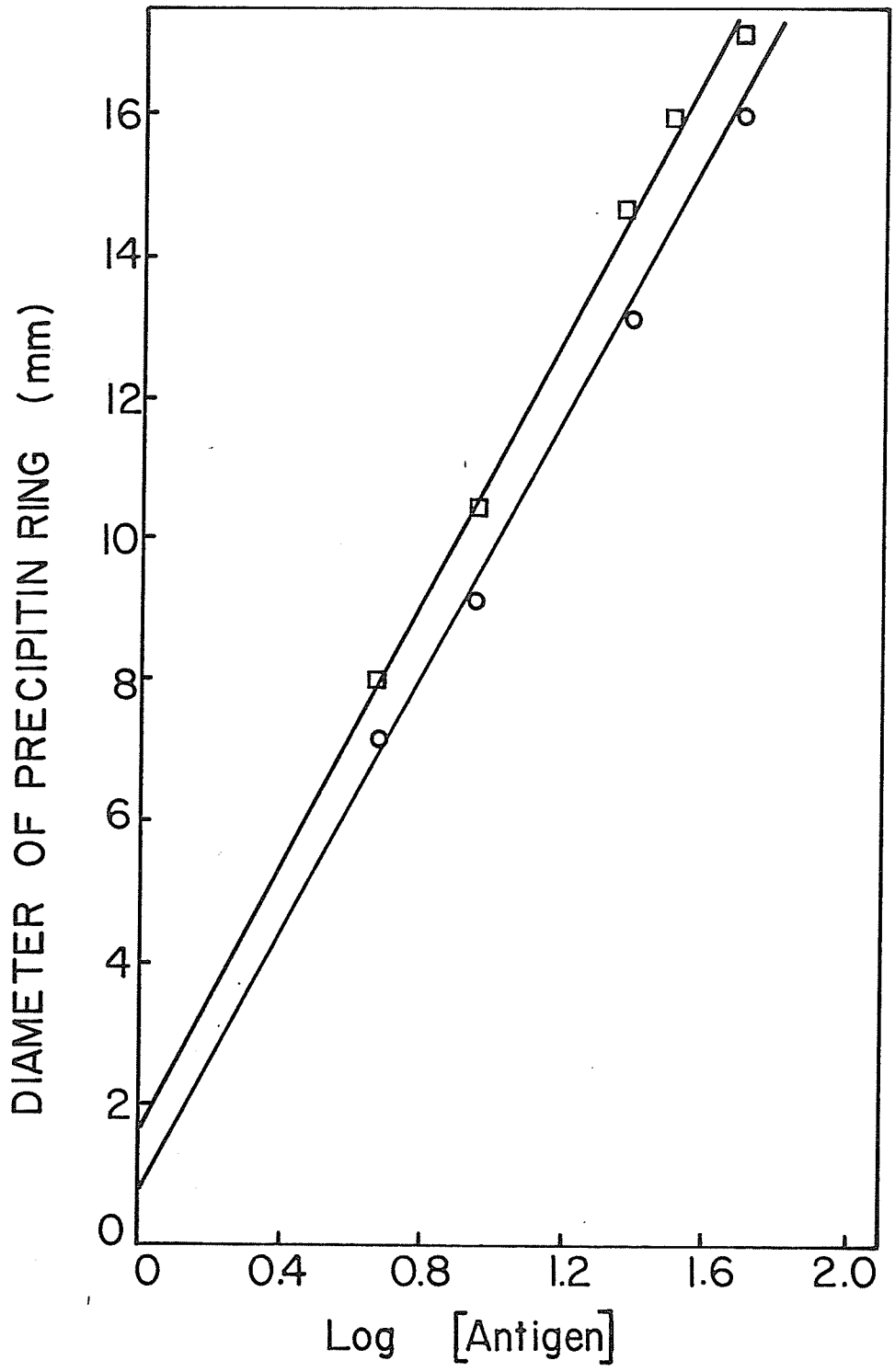
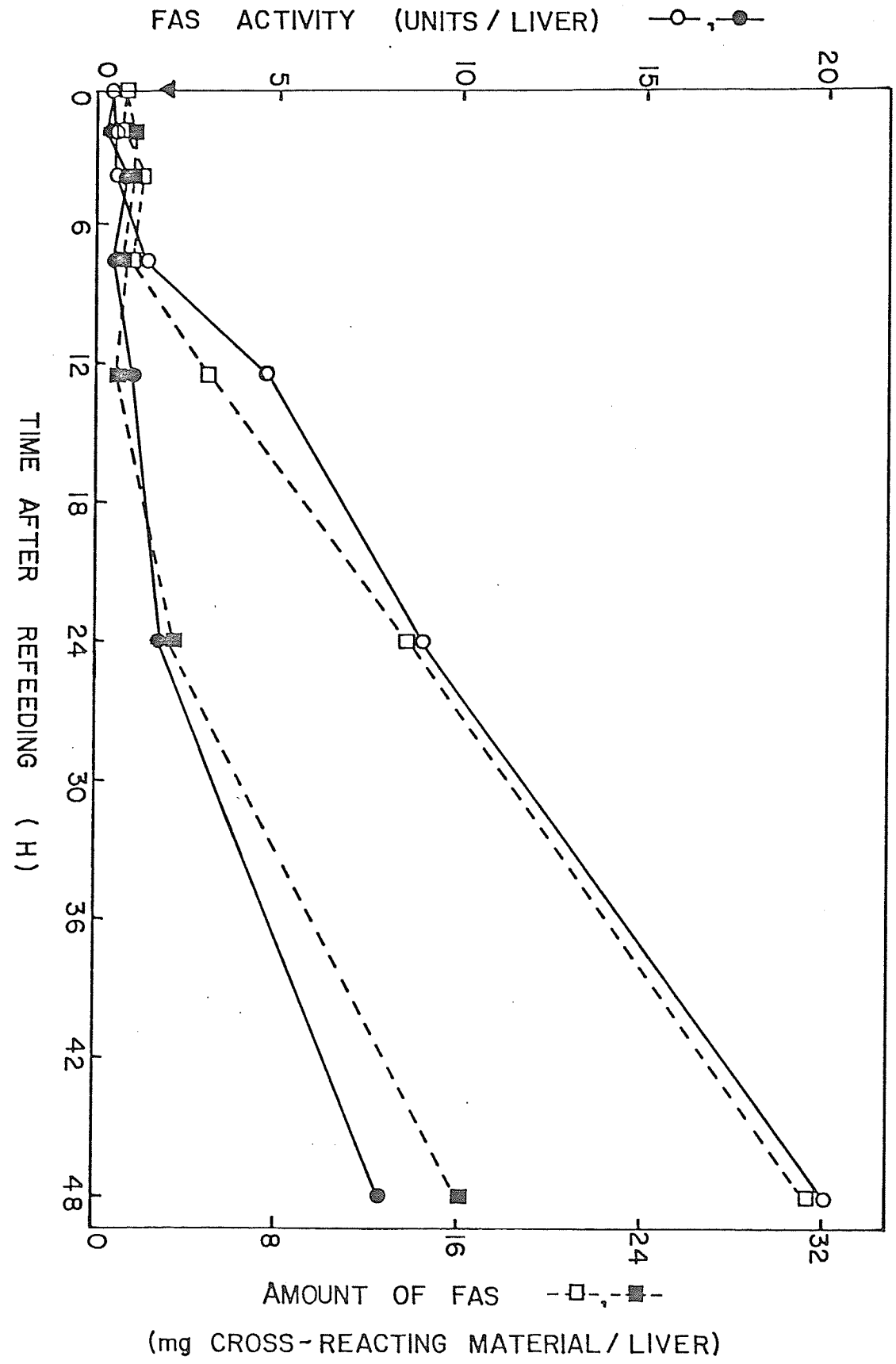

DIAMETER OF PRECIPITIN RING (mm)
0
2
4
6
8
10
12
14
16
0
0.4
0.8
1.2
1.6
2.0
Log [Antigen]

Figure 30: Effect of inflammation at 0 H of refeeding on the adaptive synthesis of FAS.

Rats were starved for 48 H and refed a fat-free diet. Inflammation was induced at 0 H after the start of refeeding (arrow). After killing at the appropriate times, FAS activity was measured spectrophotometrically, and the material precipitated by anti-FAS serum was measured by radial immunodiffusion, in a R.I.D. plate lacking DTT (Figure 27). Each point represents an average value obtained using two rats. Data for inflamed animals are indicated by the solid symbols, while the open symbols indicate non-inflamed animals. Individual values varied from this average by less than $\pm$ 12%.


FAS ACTIVITY (UNITS / LIVER) —o—,—●—
0
5
10
15
20
TIME AFTER REFEEDING (H)
0
6
12
18
24
30
36
42
48
AMOUNT OF FAS -□-,-■-
(mg CROSS-REACTING MATERIAL / LIVER)
0
8
16
24
32

2.2.6 Suppression of adaptive synthesis of FAS by experimental inflammation.

The results from the five R.I.D. experiments clearly indicate that the suppression of the adaptive synthesis of FAS by inflammation is accompanied by a decrease in material cross-reacting with anti-FAS serum, presumably FAS molecules. Table 7 summarizes the suppression of FAS activity and cross-reacting material levels by inflammation for three time intervals of refeeding. Observation of the results implies that the interactions of the two processes, expressed in our results, may be dependent upon the time intervals between the start of refeeding and inflammation and between inflammation and sacrifice. The trends are illustrated in Figure 31 and presumably are indicative of the various mechanisms interacting at the cellular level. The nature of mechanism or mechanisms that control these changes in enzyme content must involve variations in the rates of enzyme synthesis and degradation.

Figure 31: Suppression of the adaptive synthesis of FAS by experimental inflammation.

The trends summarized in Table 7 are illustrated in Figure 31 (A) for the relationship between the suppression of FAS levels and the time interval between the start of refeeding and the induction of inflammation; and in Figure 31 (B) for the relationship between the suppression of FAS levels and the time interval between the induction of inflammation and sacrifice.

The open symbols (lined) represent the suppression of activity levels for rats killed 15 H (○—○), 24 H (□—□) and 48 H (△—△) after the start of refeeding. The suppression of cross-reacting material levels is represented by the closed symbols.

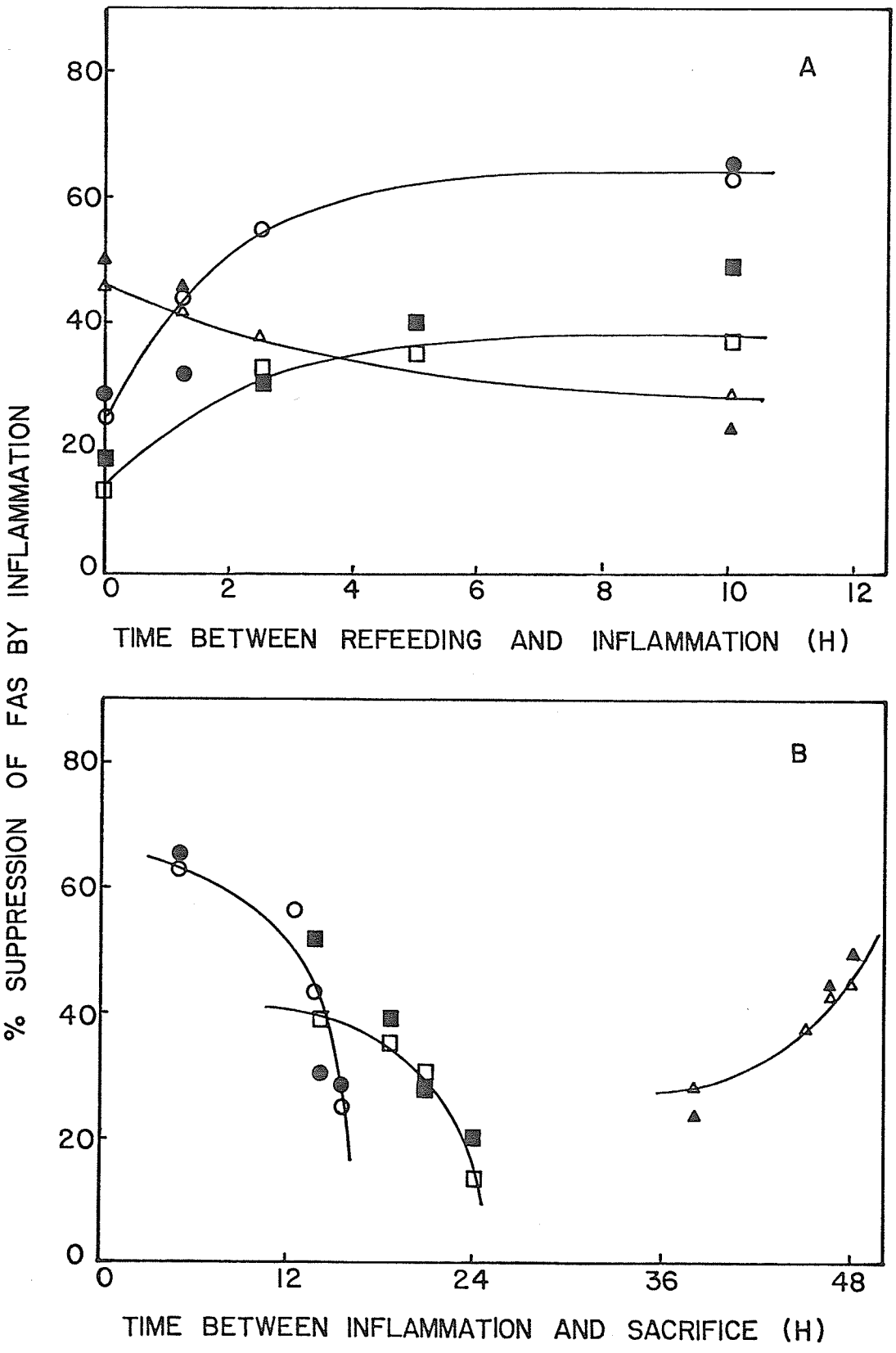

A
80
60
40
20
0
0
2
4
6
8
10
12
TIME BETWEEN REFEEDING AND INFLAMMATION (H)
% SUPPRESSION OF FAS BY INFLAMMATION
B
80
60
40
20
0
0
12
24
36
48
TIME BETWEEN INFLAMMATION AND SACRIFICE (H)

TABLE 7.

Suppression of Fatty Acid Synthetase Levels in Relation to the Interval Between Refeeding and Inflammation and Between Inflammation and Sacrifice.

Interval between refeeding and inflammation (h)	Interval between inflammation and sacrifice (h)	Suppression of FAS* Activity	Suppression of FAS* Cross-Reacting Material
(A) Rats killed 15 h after refeeding			
0	15.0	25%	29%
1.5	13.5	43	30
2.5	12.5	56	80
5.0	10.0	34	-
10.0	5.0	63	65
(B) Rats killed 24 h after refeeding			
0	24.0	14	20
1.5	22.5	46	59
2.5	21.5	33	30
5.0	19.0	35	39
10.0	14.0	38	52
(C) Rats killed 48 h after refeeding			
0	48.0	45	50
1.5	46.5	44	45
2.5	45.5	38	14
10.0	38.0	29	24

* Percentage suppression of fatty ackd synthetase levels was calculated from Figures given in Section 2.2 of the Results. The suppression represents the percent activity and cross-reacting material for FAS found in inflamed animals as compared to non-inflamed animals.

This data is also represented in Figure 31.

3. ADAPTIVE SYNTHESIS AND THE EFFECTS OF EXPERIMENTAL INFLAMMATION ON THE RELATIVE RATES OF SYNTHESIS AND DEGRADATION.

Comparisons of the rates of protein synthesis and degradation encounter a variety of difficulties depending upon the methods chosen. Ideally, experimental design should lead to determination of the rate constants of synthesis and degradation under the different conditions chosen. Complexities in studying whole animal systems make the determination of absolute rates of synthesis and degradation extremely difficult, but determination of relative rates through single-dose and double-dose isotope administration are generally considered adequate for comparative purposes. The rate of protein synthesis should measure the incorporation of label into a specific protein or protein group as a function of the change in radioactivity of the free intracellular pool of the immediate precursor. Determination of the immediate precursor of protein synthesis is complicated by segregation of the protein synthesizing apparatus, differential uptake of the label from the extracellular compartments, intracellular processes of precursor compartmentalization, etc. Other complications include protein degradation and amino acid reutilization.

Experimentally the administration of a tracer* dose of a labelled amino acid is the easiest and most convenient procedure to use. This allows determination of relative protein synthesis and protein turnover which is a measure of protein degradation. Use of a short-time interval between label administration and killing (i.e. much shorter than the assumed half-life of fatty acid synthetase), removes the danger of protein degradation and label reutilization in the studies on protein

*A tracer dose of labelled amino acid is a dose which is very small compared to the plasma pool, so that the plasma concentration of the amino acid is not significantly altered and the steady-state conditions are not disturbed.

synthesis.

For reviews on the methodologies used in studying protein synthesis and degradation see Schimke (1973); Garlick (1980); and Henshaw (1980).

3.1 Effect of Inflammation on the Relative Rates of Synthesis of FAS during Adaptive Synthesis.

The relative rates of FAS synthesis were determined by immunoprecipitation of labelled FAS and comparison of the amount of incorporation of a non-decaying* label into FAS in relation to incorporation into total protein. Two methods of immunoprecipitation were used (Volpe *et al.* 1973; Nepokroeff and Porter, 1978) which yielded similar results. Figure 32 shows a comparison of the two methods of immunoprecipitation for FAS from rats refed a fat-free diet for 62 h after 48 h starvation. In both cases the maximal precipitation of labelled protein occurred with 1.4 mg of anti-FAS antiserum and 45-60 units of FAS.

Figure 33 compares the suppression of FAS activity and relative rate of synthesis by inflammation at the start of refeeding. Rats were starved for 48 h and refed a fat-free diet for up to 48 h. Labelled leucine was administered 1 h prior to killing and the rats treated as described in Experimental Procedures. The adaptive synthesis of FAS with refeeding for 48 h resulted in a 25-fold increase in activity and a 12-fold maximal increase in the relative rate of synthesis occurring at 24 h refeeding. Inflammation caused a 65% decrease in FAS activity and a 60% decrease in the relative rate of

*"Non-decaying label" refers to a labelled compound injected a short-time prior to killing so that incorporation into the protein occurs but no significant decay of the label from the protein (i.e. through protein degradation) can occur.

Figure 32: Immunoprecipitation of labelled FAS by two different methods.

Animals were starved for 48 H and refed a fat-free for 62 H prior to killing.

(A) Varying amounts of 105,000 g supernatant were added to 50 µl of partially purified antiserum. The samples were incubated as described in Experimental Procedures and washed by pelleting through sucrose. The equivalence point is approximately 1400 µg of antiserum for 60 units of FAS.

(B) Varying amounts of partially purified anti-FAS antiserum were added to 50 µl of purified FAS (22-40 % ammonium sulfate fraction) as described in Experimental Procedures and washed three times with saline. The equivalence point is approximately 1400 µg of antiserum to 45 units of FAS.

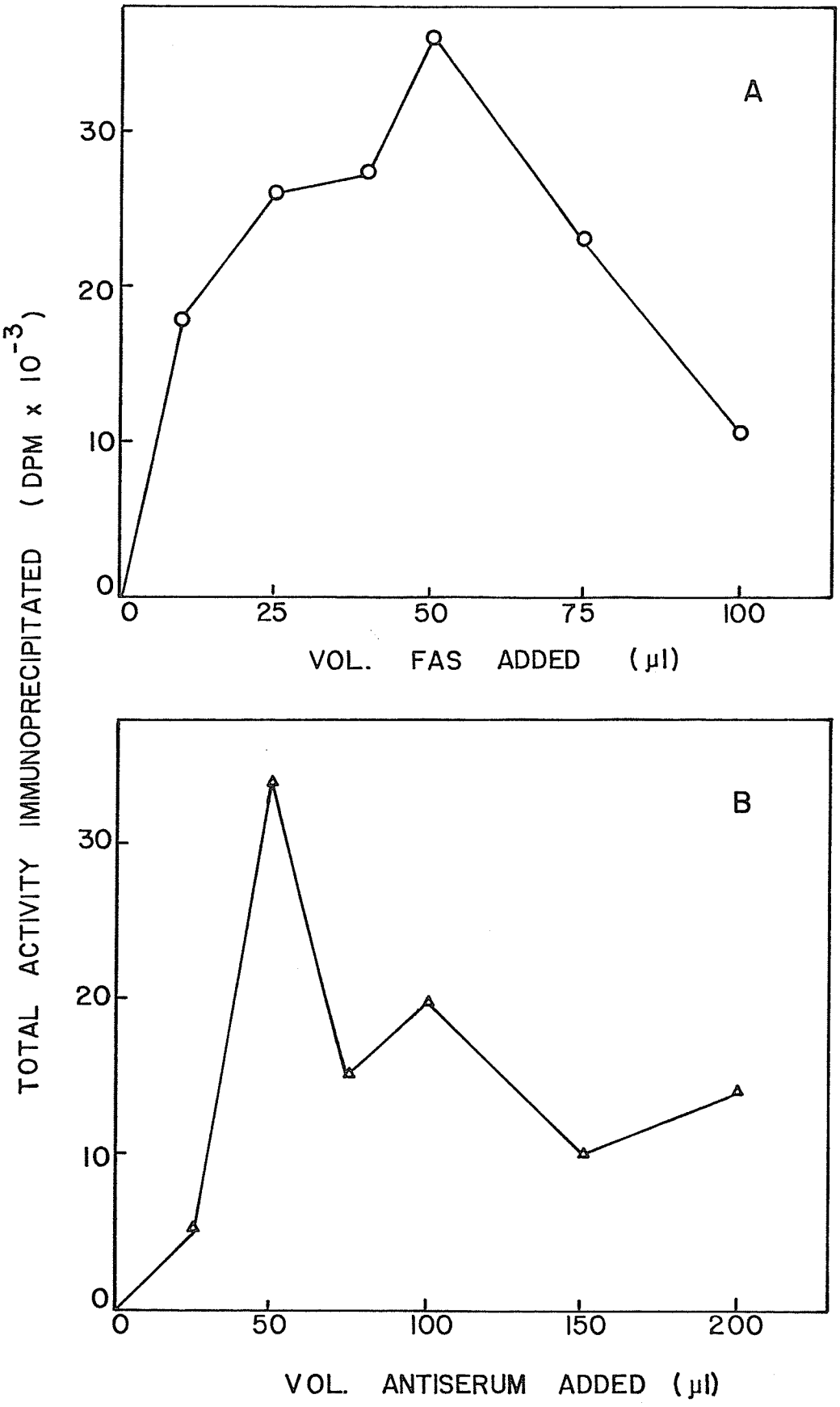
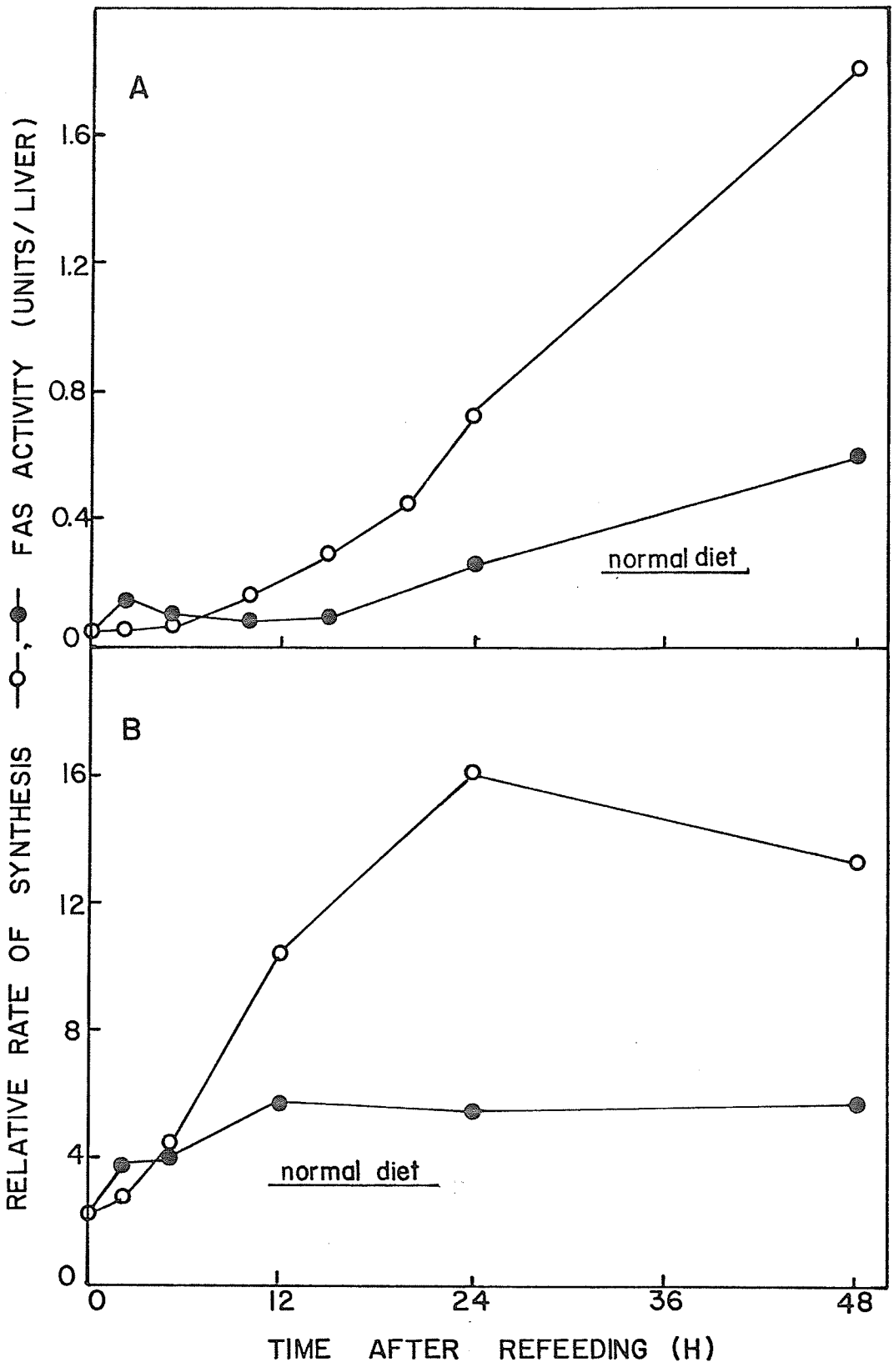

A
30
20
10
0
0
25
50
75
100
VOL. FAS ADDED (µl)
TOTAL ACTIVITY IMMUNOPRECIPITATED (DPM x 10^{-3})
B
30
20
10
0
0
50
100
150
200
VOL. ANTISERUM ADDED (µl)

Figure 33: Effect of inflammation on the adaptive synthesis of FAS.

Rats were starved for 48 H and refed a fat-free diet. Inflammation was induced 0 H after the start of refeeding. After killing at appropriate times, FAS activity (A) and the Relative Rate of FAS Synthesis (B) were determined as described in Experimental Procedures. Each point represents an average value obtained using 2 rats. Data for inflamed animals is indicated by solid symbols and for non-inflamed animals is indicated by open symbols. Levels for animals maintained on a normal diet is indicated by the line labelled normal diet.

FAS activity is given as micromoles of palmitate produced per minute per liver. Individual values varied from this average by less than $\pm$ 12%.


A
B
1.6
1.2
0.8
0.4
0
16
12
8
4
0
normal diet
normal diet
0
12
24
36
48
TIME AFTER REFEEDING (H)
RELATIVE RATE OF SYNTHESIS —○—, —●— FAS ACTIVITY (UNITS/LIVER)

enzyme synthesis.

Figures 34 and 35 represent the changes in activity and synthesis for rats refed up to 162 h, with inflammation induced at 62 h of refeeding. The data from Figure 33 is also included for comparison. At long times after refeeding FAS levels begin to decline to normal levels. The induction of inflammation at the start of this decline caused the levels of FAS to drop below the level in normal animals (refed a standard diet). The decline in activity in both inflamed and non-inflamed animals was accompanied by a corresponding decline in the relative rates of enzyme synthesis. After 162 h of refeeding the activity in the inflamed animals decreased to 25% that of the non-inflamed animals while the relative rate of synthesis was 50% that of the non-inflamed animals.

The influence of protein degradation on the changes in FAS levels is implicated by two observations from the changes in relative synthesis. Figure 35 shows the suppression of the relative rate of synthesis by inflammation cannot totally account for the suppression of activity at 162 h of refeeding. In Figure 33 the lag period observed (0-5 h) in the induction of enzyme activity was absent for the induction of enzyme synthesis for both inflamed and non-inflamed animals. The early rise in relative synthesis for inflamed animals implies a cellular mechanism of protein degradation must be involved or an observable increase in activity should be apparent.

3.2 Effects of Experimental Inflammation on the Relative Rates of Degradation of FAS.

Determination of the rate of degradation of a protein encounters the same difficulties outlined for the determination of protein synthesis.

Figure 34: Effect of inflammation upon the adaptive synthesis of FAS.

Rats were starved for 48 H and refed a fat-free diet. Inflammation was induced at 0 H and 62 H (arrows) after the start of refeeding. After killing at appropriate times, FAS activity was determined as described in Experimental Procedures. Each point represents an average value obtained using 2 rats. Data for inflamed animals is indicated by solid symbols and for non-inflamed animals is indicated by open symbols.

FAS activity is given as micromoles NADPH oxidized per minute per liver.

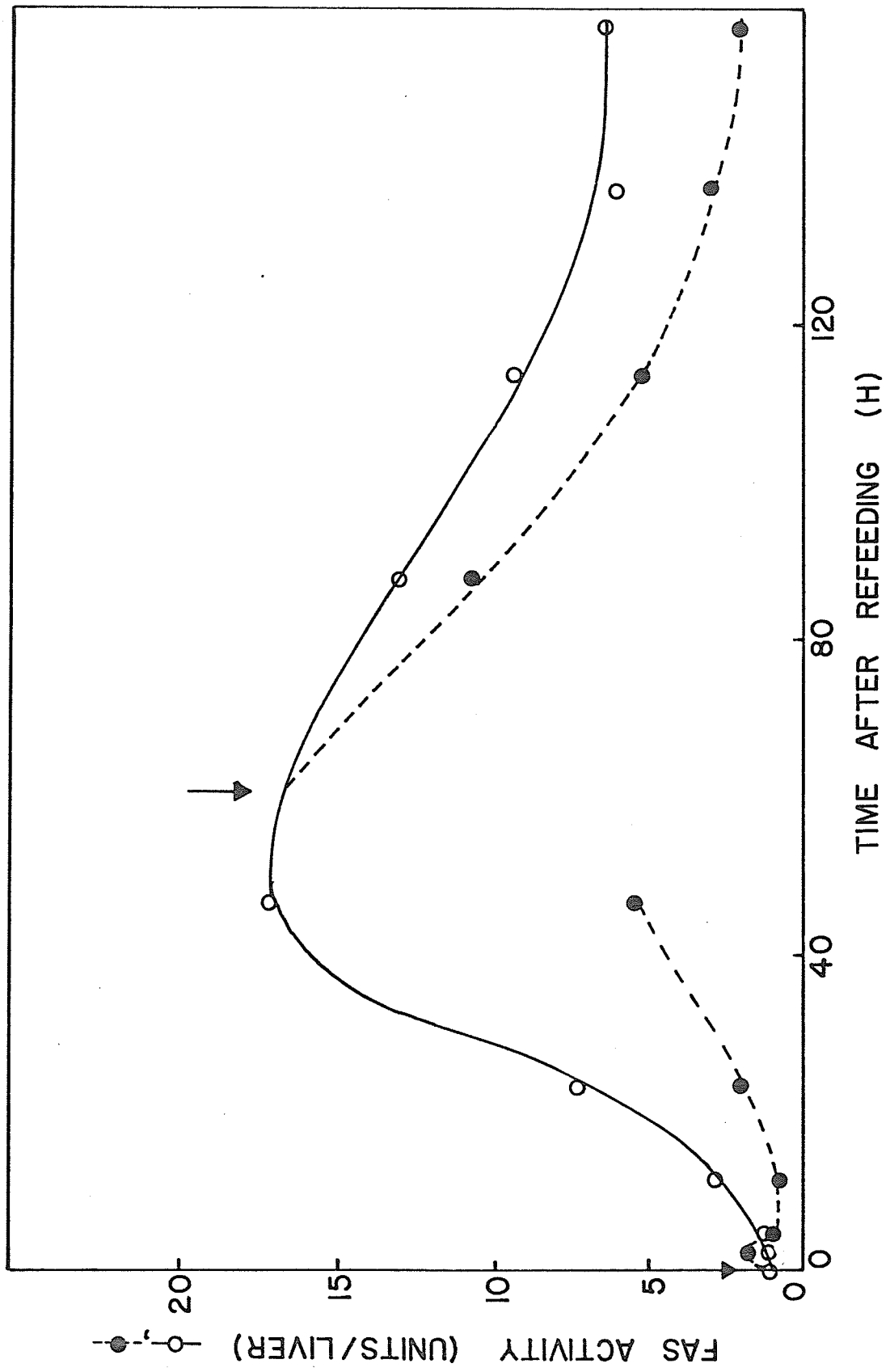
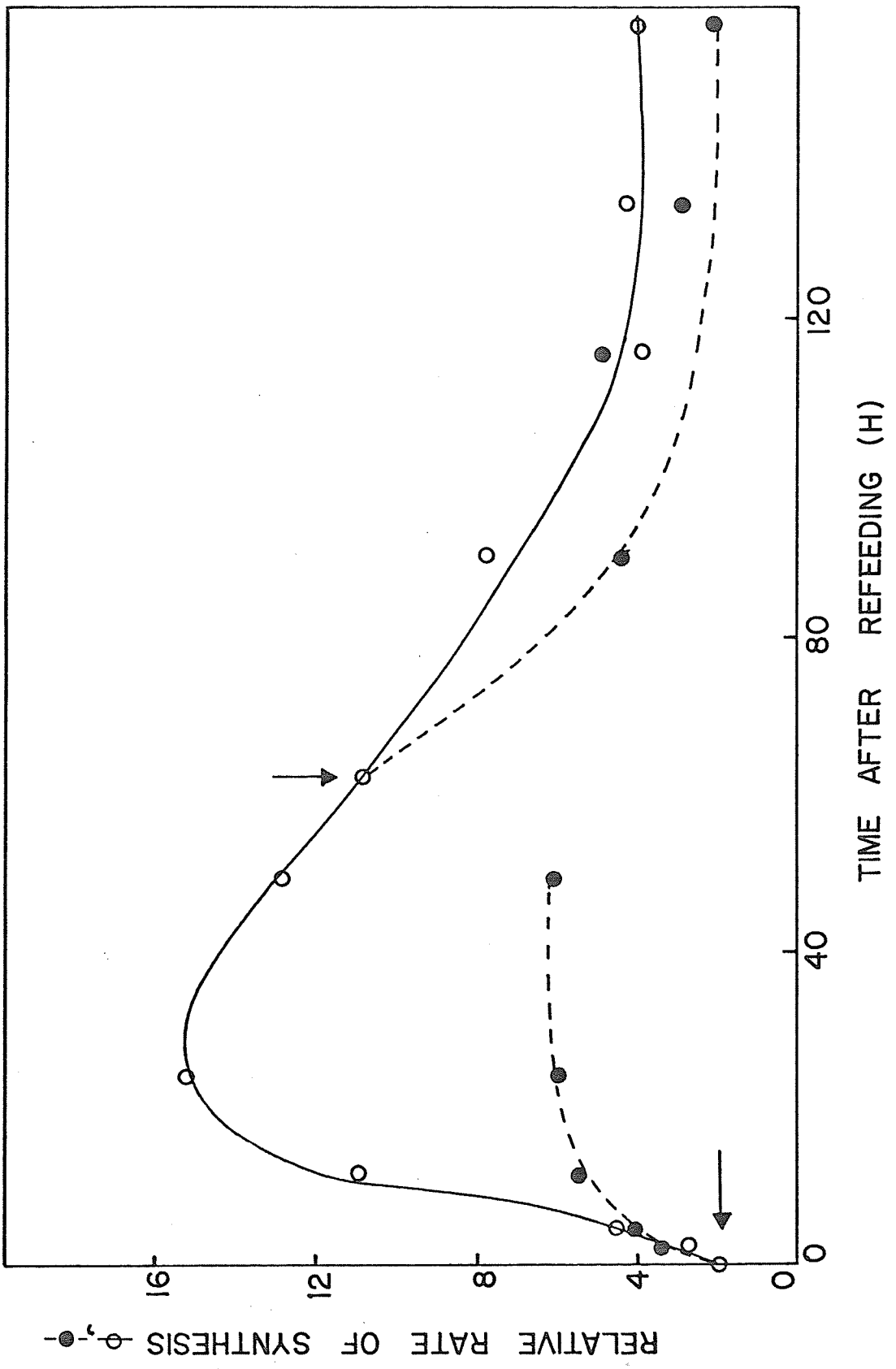

FAS ACTIVITY (UNITS/LIVER)
0
5
10
15
20
0
40
80
120
TIME AFTER REFEEDING (H)

Figure 35: Effects of inflammation upon variations in the Relative Rates of FAS Synthesis during the adaptive synthesis of FAS. Other details are given in the legend to Figure 34.


RELATIVE RATE OF SYNTHESIS
0
4
8
12
16
0
40
80
120
TIME AFTER REFEEDING (H)

The use of a decaying* label administered at a relatively long time prior to killing increases the danger of amino acid reutilization. About 20% of the amino acids released in the body by protein breakdown are oxidized and excreted while the remaining 80% are resynthesized into new protein in the same tissue or, via the bloodstream, in other tissues (Waterlow *et al.* 1978). This problem manifests itself differently for different amino acids and leads to label reincorporation into protein while degradation measurements are being made. Thus single-isotope methods used for the determination of the actual value, and the degree of this overestimation is a function of the extent to which the amino acid is subject to reutilization. This problem becomes more serious during periods of rapid protein turnover but the methods used in this study are applicable on a comparative basis when conditions are approaching a steady-state.

3.2.1 Effect of inflammation on the half-life of FAS during adaptive synthesis.

The half-life of a protein is the time required for one-half of the protein molecules present initially in the pool to be lost (degraded). It can be determined graphically as in figure 36 or mathematically by the relationship:

$$t_{\frac{1}{2}} = \frac{\ell n\ 2}{k_d}$$

which is derived from the rate equation:

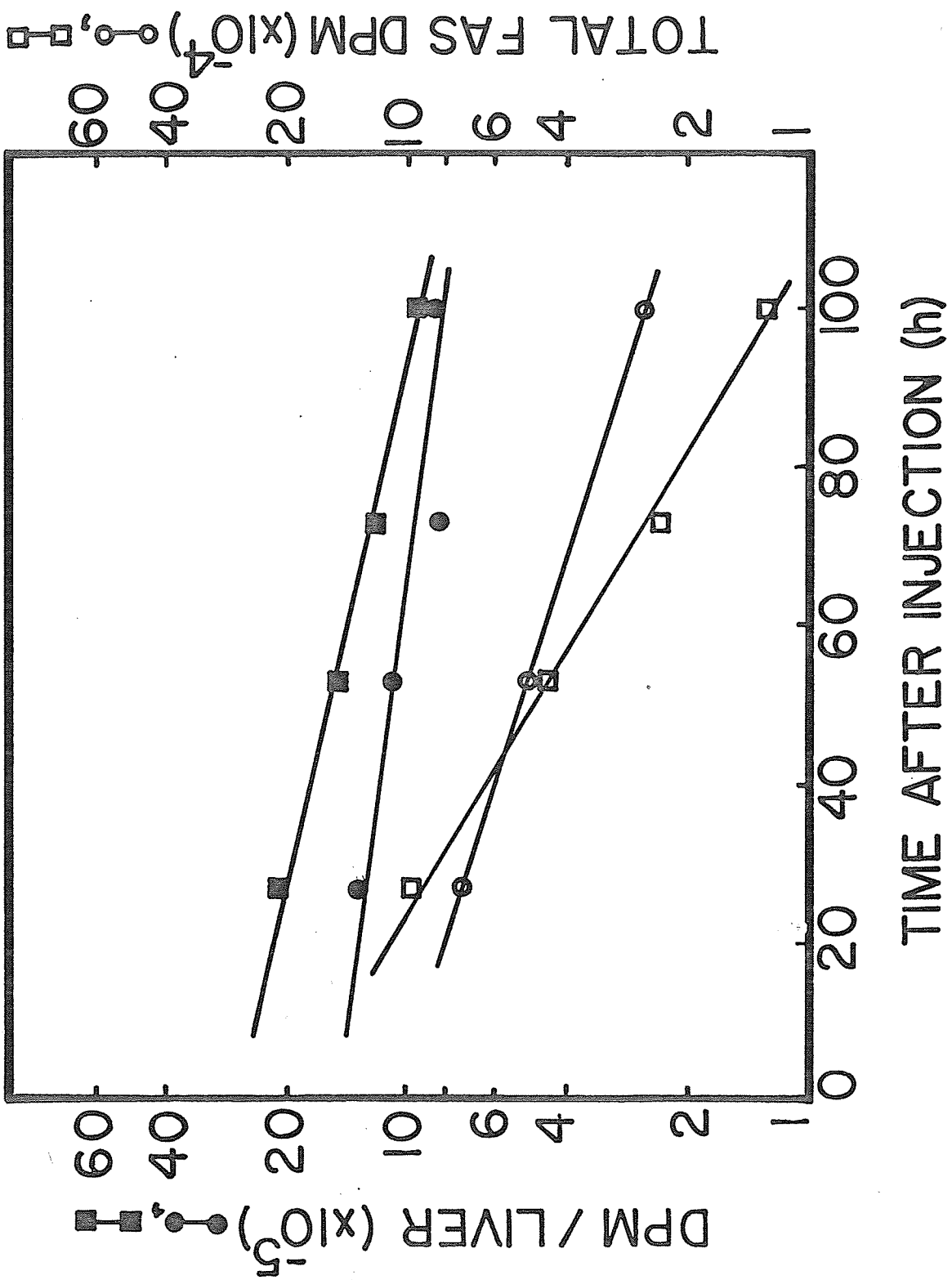
$$dE_{(t)}/d(t) = -k_d E_{(o)}$$

where $E_{(o)}$ is the initial number of enzyme molecules present, $E_{(t)}$ is

*Decaying label is a labelled compound injected a long-time prior to killing so that incorporation into the protein has occurred and significant decay of label from the protein, through degradation, is occurring.

Figure 36: Effects of inflammation on the decay of FAS and total protein during refeeding of a fat-free diet.

Rats were starved for 48 H and refed a fat-free diet. Inflammation was induced at 62 H after the start of refeeding and the rats killed at the appropriate times after the start of inflammation. The levels of decaying label in FAS and total protein were determined as described in Experimental Procedures. Data for inflamed animals is indicated by (□—□) for FAS and (■—■) total protein and for non-inflamed animals by (○—○) for FAS and (●—●) for total protein.


DPM / LIVER (x10⁻⁵)
TOTAL FAS DPM (x10⁻⁴)
TIME AFTER INJECTION (h)
0
20
40
60
80
100
1
2
4
6
10
20
40
60

the number remaining after time (t) and k_d is the rate constant of degradation.

To determine the effect of inflammation on the half-life of FAS during adaptive synthesis, the time interval of 62 to 162 h of refeeding was used. Inflammation was induced at 62 h after the start of refeeding. During this time period the activity (Figure 34) and the relative rates of synthesis (Figure 35) were approaching a steady-state condition. Thus the observed depression in enzyme half-life by inflammation is a good estimation of the effect of inflammation on enzyme degradation. The results are presented in Figure 36 and Table 8. Inflammation decreased the half-life of FAS by 50% while decreasing the total protein half-life by 15%. These results are in agreement with earlier studies which found degradative increases occurring while the rates of synthesis were decreasing (see Volpe and Vagelos, 1974). This led to the proposal that changes in degradation were controlled by variations in the rate of synthesis.

3.2.2 Variations in the turnover and turnover-index during adaptive synthesis and inflammation.

During the non-steady state conditions occurring at the beginning of the refeeding period (i.e. when the rates of synthesis are rapidly changing) determination of protein half-lives are difficult due to differential enzyme reutilization, etc. The double-isotope method allows determination of the turnover and turnover-index of FAS which, with the knowledge of the relative synthesis rates, allows an estimation of the relative degradation taking place. Comparison of the loss of incorporated radioactivity from the decaying label, to the rate of incorporation of a non-decaying label injected shortly before killing, allows determination of the turnover of a specific protein. This

TABLE 8.

Half-Lives and Relative Rates of Degradation of Fatty Acid Synthetase*

	Half-Life		Relative k_d	
	Non-Inflamed	Inflamed**	Non-Inflamed	Inflamed**
PROTEIN	72 h	60	0.010 (h^{-1})	0.012
FAS	54	26	0.015	0.027

*Calculated from Figure 36. Rats were refed a fat-free diet for 62 - 162 h after 48 h of starvation.

**Rats were given a subcutaneous injection of oil of turpentine at 62 h of refeeding.

ratio is an adequate measure of the relative degradation occuring at a specific time and can be used to compare two sets of animals. Table 9 presents the data from rats refed up to 162 h with turpentine injections at 0 h and 62 h refeeding. The results indicate that inflammation causes a rapid increase in turnover in the first 12 h after refeeding. The nature of this relationship is complex and is better represented by the turnover index given in Table 10 (see Ganschow and Schimke, 1969; Ciaranello and Axelrod, 1973).

The turnover index allows estimation of the relative synthesis and degradation of a specific protein relative to the average turnover of proteins from the same tissue.

$$\text{T.I.} = \frac{\text{DPM from non-decaying label for FAS}}{\text{DPM from decaying label for FAS}} \times \frac{\text{DPM from decaying label for total protein}}{\text{DPM from non-decaying label for total protein}}$$

Rearranging, the turnover index is made up of two parameters:

$$\text{T.I.} = \frac{k_s(\text{FAS})}{\frac{\text{DPM from decaying label for FAS}}{\text{DPM from decaying label for Total Protein}}}$$

which is a representation of the relative degradation of FAS compared to that for total protein.

A rapid turnover will give a high turnover index and is dependent upon both synthesis and degradation. After 12 h of refeeding the relative rate of synthesis of FAS for non-injected rats was twice that of rats inflamed with turpentine. The turnover index for the same time interval was approximately twice that for the inflamed animals indicating that the increase in turnover was due to an apparent increase

Table 9.

Effect of Inflammation and Adaptive Synthesis on the Turnover of Fas and Total Soluble Protein.

Duration of fat-free feeding (H).[a]	Time of turpentine injection[b]	FAS Turnover[c]		Total Protein Turnover[c]	
		Non-Inflamed	Inflamed	Non-Inflamed	Inflamed
0	0 Hr after the start of refeeding	0.509	-	0.799	-
12		0.612	1.24	1.03	1.15
24		0.833	0.963	1.34	1.13
90	62 Hr after the start of refeeding	0.216	0.195	0.338	0.231
116		0.191	0.526	0.256	0.297
136		0.537	0.671	0.256	0.332
162		0.925	0.786	0.428	0.446

[a] Rats were starved for 48 H prior to refeeding a fat-free diet.

[b] A subcutaneous injection of oil of turpentine was given at either 0 or 62 H after the start of refeeding.

[c] $\text{Turnover} = \text{Fas} \frac{\text{DPM } ^{14}C}{\text{DPM } ^{3}H}$ Or

$= \text{Total Protein} \frac{\text{DPM } ^{14}C}{\text{DPM } ^{3}H}$

Table 10.

Effect of Inflammation and Adaptive Synthesis on the Turnover Index of FAS.

Duration of fat-free feeding (H).[a]	Time of turpentine injection[b]	Turnover-Index	
		Non-Inflamed	Inflamed
0	0 Hr after the start of refeeding	0.637	-
12		0.596	1.08
24		0.620	0.856
90	62 Hr after the start of refeeding	0.640	0.843
116		0.745	1.77
136		2.09	2.02
162		2.16	1.76

[a] Rats were starved for 48 H prior to refeeding a fat-free diet.

[b] A subcutaneous injection of oil of turpentine was iven at either 0 or 62 H after the start of refeeding.

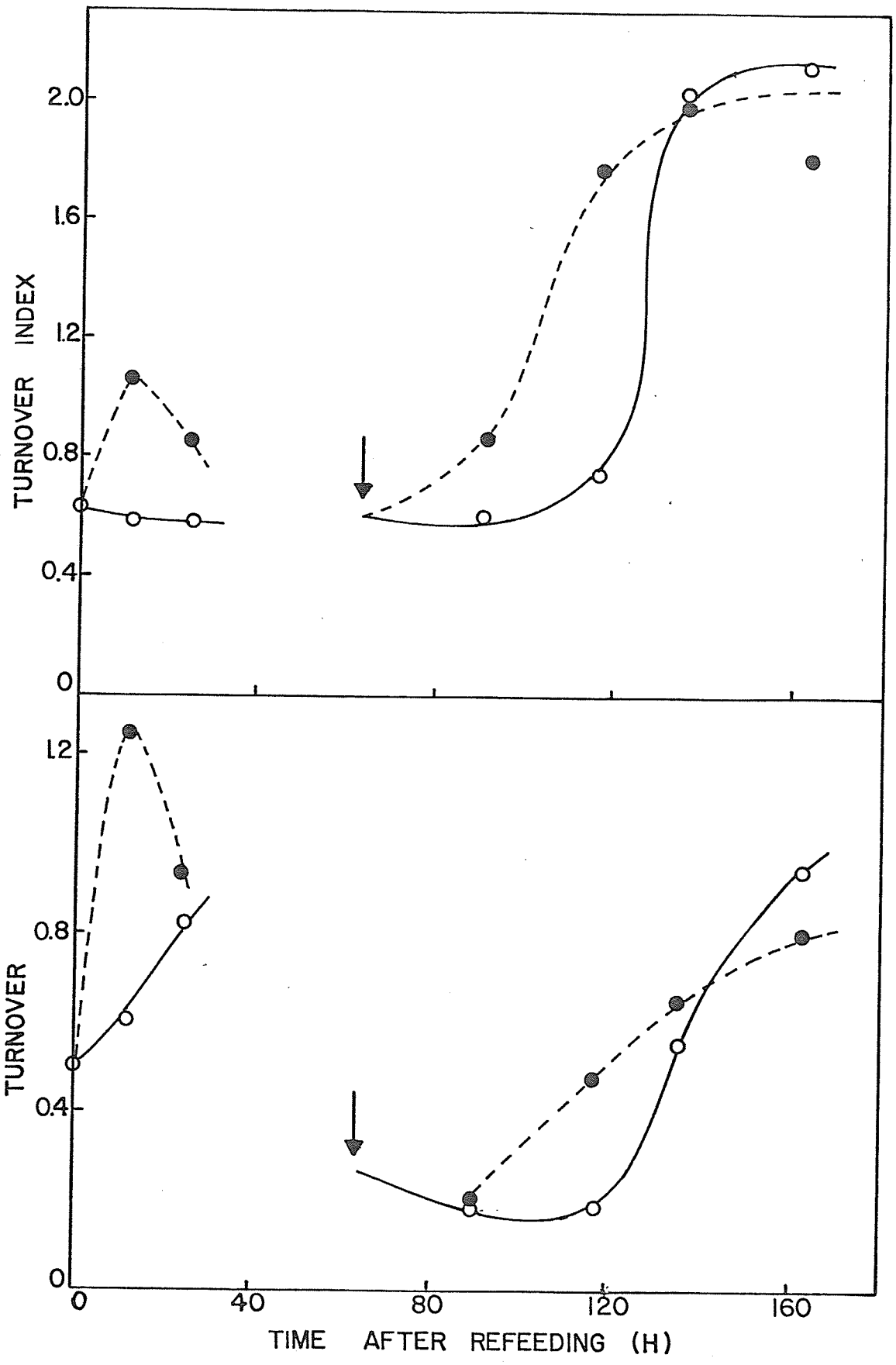
[c] Turnover Index = $\frac{\text{DPM FAS } {}^{14}C/{}^{3}H}{\text{DPM Total Protein } {}^{14}C/{}^{3}H}$

in the relative degradation of FAS. For the experiment where inflammation was induced at 62 h after the start of refeeding, an initial higher turnover and turnover index was observed for inflamed animals. At longer times after inflammation the turnover and turnover-index appeared identical.

The data from Tables 9 and 10 is also presented in Figure 37.

Figure 37: Turnover index and Turnover of rat liver fatty acid synthetase.

The data from Tables 9 and 10 is represented graphically showing the variations in the Turnover index (top) and the Turnover (bottom) for non-inflamed rats (O——O) and for inflamed rats (●——●). Inflammation was induced at 62 H and 0 H after the start of refeeding.


TURNOVER INDEX
2.0
1.6
1.2
0.8
0.4
0
TURNOVER
1.2
0.8
0.4
0
0
40
80
120
160
TIME AFTER REFEEDING (H)

4. EFFECTS OF ADAPTIVE SYNTHESIS AND EXPERIMENTAL INFLAMMATION ON THE LEVELS OF TRANSLATABLE MESSENGER RNA FOR FATTY ACID SYNTHETASE.

The data in the previous sections indicated that the observed variations in the levels of FAS during adaptive synthesis and inflammation were due to changes in the relative rates of synthesis and degradation of the enzyme. The mechanisms of the changes in degradation are difficult to identify and were not investigated further. As was discussed in the Historical section the importance of degradation in cellular control is becoming more and more apparent.

The variations in the rates of synthesis of FAS may be due to an increase in the levels of translatable message through increased transcription or processing of heteronuclear RNA, increased translation of already present message or both. To investigate this question the poly(A)-containing RNA was isolated, partially characterized and translated in a cell-free rabbit reticulocyte lysate system.

4.1 Isolation and Partial Characterization of the Poly(A)-containing Messenger RNA from Rat Liver.

Poly(A)-containing messenger RNA was isolated as described in the Experimental Procedures. Total cytoplasmic, magnesium-precipitable RNA was isolated according to Palmiter (1974) and subjected to a phenol-chloroform extraction as described. The RNA extracted was dissolved in a high salt buffer and applied to an oligo-(dT) cellulose column to isolate the poly(A)-containing RNA for translation. Current evidence indicates that functional messenger-RNA isolated from the cytoplasm contains a poly-adenylated "tail" (Harpold *et al.*, 1981).

Table 11 presents the data from the last three steps of a typical purification of poly(A)-containing RNA from rats treated as described. The results are presented as the number of absorbance units at 260 nm

Table 11.

Purification of RNA from Starved, Refed and Inflamed Rats.

RNA purification step	Starved 48 H[a]			Starved 48 H and refed 12 H.[a] Non-inflamed			Starved 48 H and refed 12 H.[a] Inflammation at 0 H of refeeding		
	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}	A_{280}	A_{260}/A_{280}
Magnesium precipitated polysomes	133.5 1034.8**	70.9	1.88	98.8 1833.7**	54.9	1.80	139.9 1455.2**	75.5	1.85
High-salt buffer	56 433.7**	26.3	2.17	43.6 809.6**	20.6	2.12	55.5 577.2**	27.0	2.06
Poly(A)-mRNA	2.38 18.45**	0.889	2.10	2.18 40.5**	1.01	2.2	2.26 23.5**	0.92	2.08

[a] Animals were starved for 48 H and refed a fat-free diet for 12 H, and killed at the appropriate times.

** All values are given as per gram of liver unless indicated by a double asterisk, which indicates a value per total liver weight.

and 280 nm per gram wet weight of liver and total per liver. (One A_{260} unit represents approximately 50 µg RNA as per Haines *et al.* 1974). The final poly(A)-containing mRNA was approximately 4% of the amount applied to the oligo(dT)-cellulose column and consistently had an A_{260}/A_{280} ratio of 2.0-2.2. The increase in total poly(A)-mRNA for refed rats without inflammation is due to a consistently larger liver weight. This increased weight is partially due to a rapid rise in the amount of glycogen deposited during refeeding without inflammation (see next section of results).

Figure 38 illustrates the oligo(dT)-cellulose chromatography of the total cytoplasmic RNA from rats starved for 48 h and refed a fat-free diet for 12 h (as in Table 11). The fractions under the bars were precipitated with ethanol (overnight at -20°C) and translated for mRNA activity (presented in a later section).

Partial characterization of the poly(A)-containing mRNA was accomplished by electrophoresis and sucrose density centrifugation. Sucrose density gradient centrifugation under non-denaturing conditions was performed as described in the Experimental Procedures. To overcome the problem of RNA aggregation under non-denaturing conditions the poly(A)-mRNA samples were heated to 70°C for 1 min and rapidly cooled to 4°C prior to centrifugation. Figure 39 presents the results from a 5-20% sucrose density gradient centrifugation in 0.1 M sodium acetate, pH 5.5 containing 1 mM EDTA and 0.5% SDS. The poly(A)-containing sample was isolated from two sets of rats subjected to the experimental conditions described in the figure legend. The sedimentation profile for the non-inflamed rats is similar to that obtained by Nepokroeff and Porter (1978). The profile for the sample from rats inflamed at 0 h

Figure 38: Oligo(dT)-cellulose chromatography of total cytoplasmic, magnesium-precipitable DNA.

Rats were starved for 48 H and refed a fat-free diet for 12 H prior to killing. RNA was extracted as described and applied to an oligo(dT)-cellulose column (approx. 50 A_{260} units were applied to 0.5 g of oligo(dT)-cellulose). The bound poly(A)-containing RNA was eluted in low salt (0.01 M Tris) buffer, as described in Experimental Procedures. Both sets of fractions under the bars were collected and precipitated overnight with ethanol.

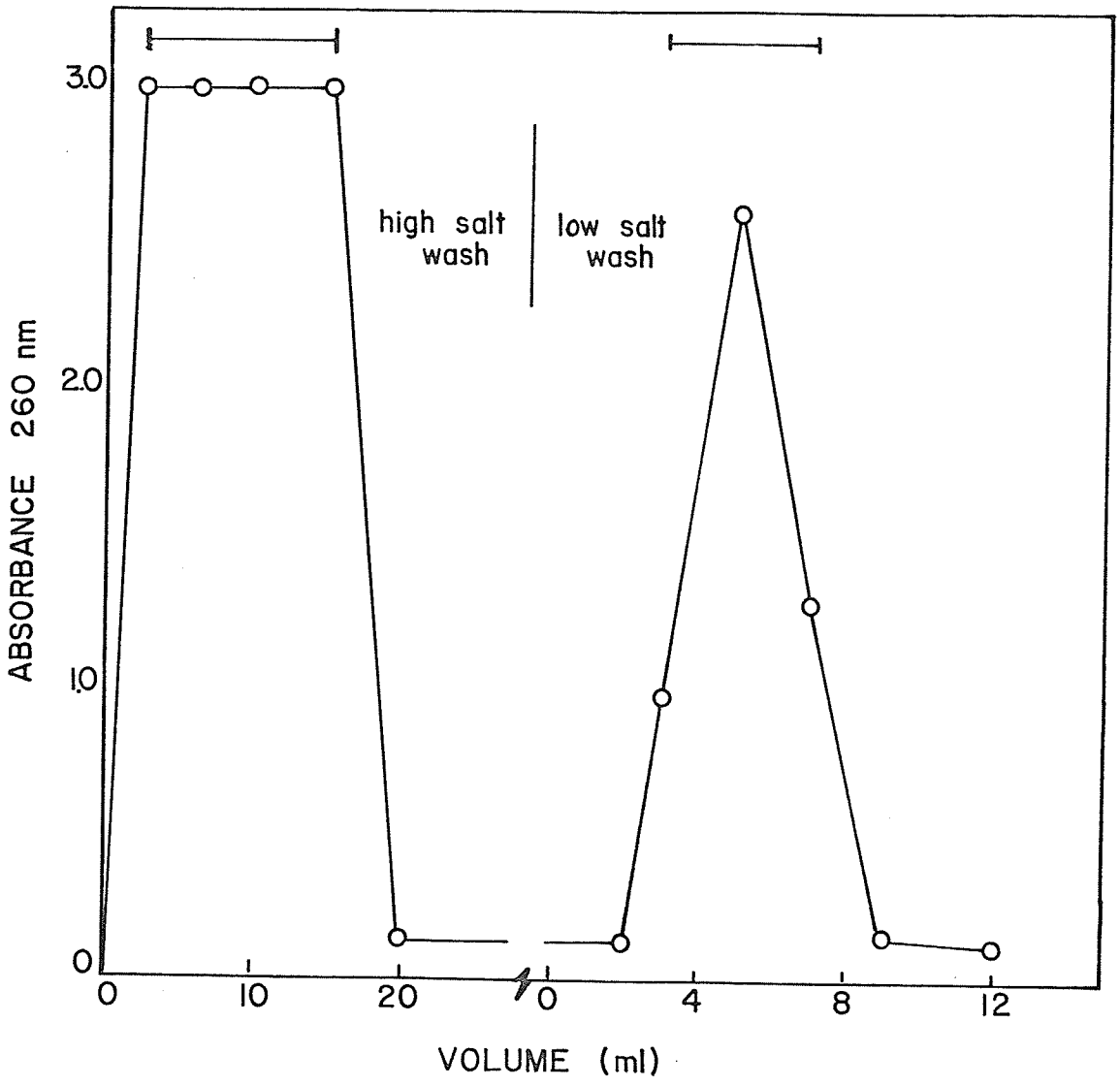
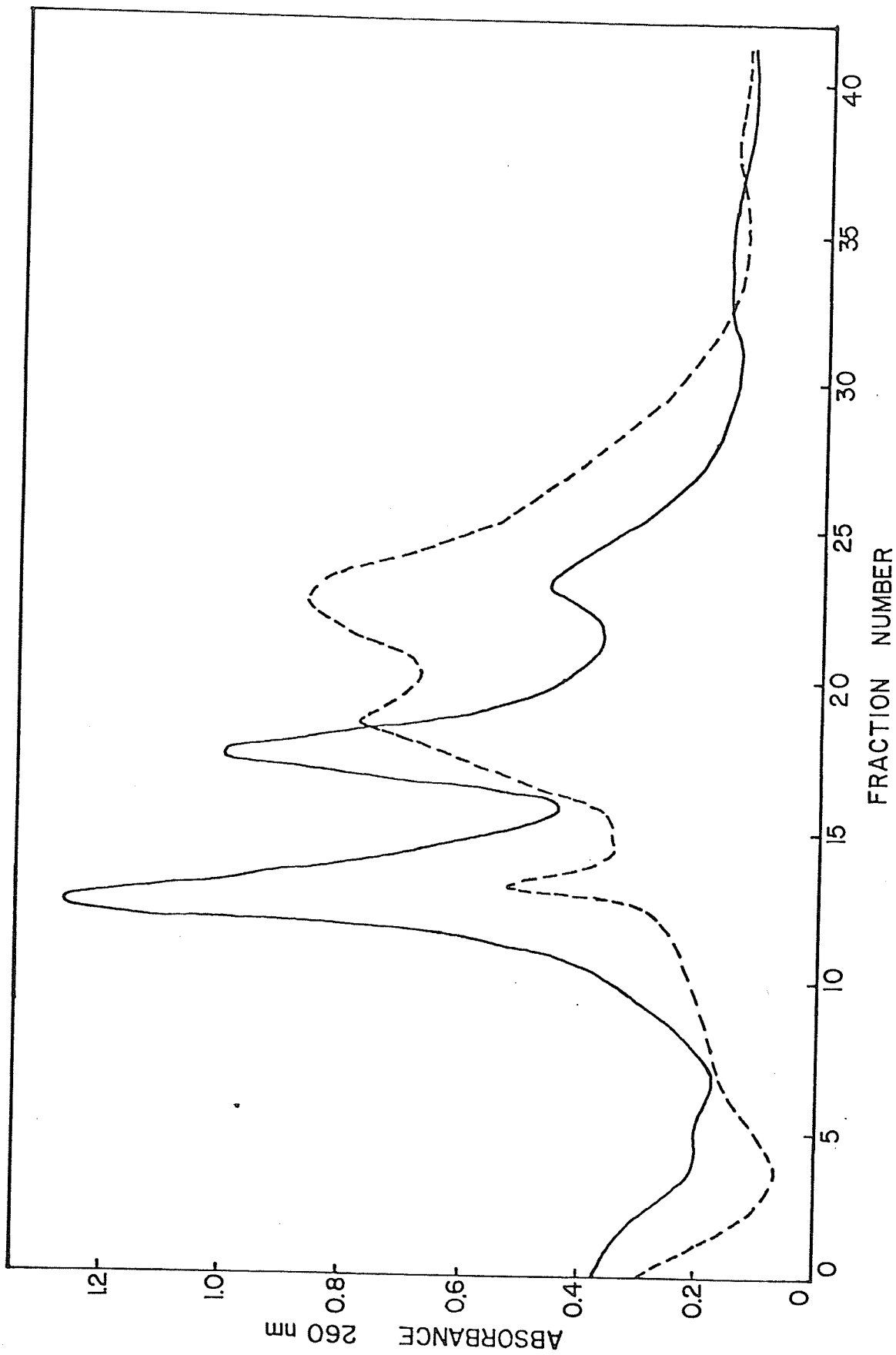

3.0
2.0
1.0
0
ABSORBANCE 260 nm
high salt wash
low salt wash
0
10
20
0
4
8
12
VOLUME (ml)

Figure 39: Sedimentation profile of poly(A)-mRNA in a 5-20 % sucrose density gradient.

Approximately 200 µg of poly(A)-mRNA from rats starved for 48 H and refed for 12 H (non-inflamed ———, inflamed at 0 H of refeeding-----) was sedimented through a 5-20 % sucrose gradient in 0.1 sodium acetate, pH 5.5 containing 1 mM EDTA and 0.5 % SDS. The samples were centrifuged at 35,000 rpm for 5.5 H in an SW 41 rotor, at 20° C.


ABSORBANCE 260 nm
0
0.2
0.4
0.6
0.8
1.0
1.2
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

of refeeding had an increase in the lower molecular weight RNAs and a decrease in the larger sized mRNA population. These results may imply the existence of a cellular control acting on different sized populations of messenger RNA under different environmental conditions (see Discussion). The difference was also observed in a 10-25% sucrose gradient for rats starved for 48 h and refed a fat-free diet for 4 h with an injection of turpentine at 0 h of refeeding for the inflamed animals. Figure 40 presents the results and includes ribosomal RNA standards for *E. coli* and calf liver which were run simultaneously.

The poly(A)-mRNA was also partially characterized by electrophoresis in agarose-urea gels. Low percentage polyacrylamide gels were also attempted with little success (by the method of Peacock and Dingman, 1968). Agarose gel electrophoresis in 6.0 M urea at an acid pH has several advantages over polyacrylamide gel electrophoresis. These include the absence of polymerizing agents that may interact with RNA, physical ease of handling and denaturing conditions that minimize aggregation of RNA. Although not fully denaturing, agarose-urea gels provide an adequate estimation of the sizes of the mRNA present (for a discussion of gel electrophoresis of RNA under denaturing conditions see Lehrach *et al.* (1977). Figure 41 illustrates the electrophoresis of poly(A)-mRNA samples isolated as described and run on 1.5% agarose gels. The *E. coli* and calf liver ribosomal RNA standards were run as indicated. Figure 42 represents the standard curve of molecular weight versus relative mobility of the standards with the approximate size of the larger mRNA bands identified by arrows. The observed bands are similar to those identified by Zehner *et al.* (1980)

Figure 40: Sedimentation profile of poly(A)-mRNA in a 10-25 % sucrose density gradient.

Approximately 100 µg of poly(A)-containing mRNA from rats starved for 48 H and refed a fat-free diet for 4 H (non-inflamed————, inflamed at 0 H of refeeding-----) was sedimented through a 10-25 % sucrose gradient in 10 mM HEPES, pH 7.5 containing 1 mM EDTA and 1 mM NaCl. The samples were centrifuged at 25,000 rpm for 24 H in an SW 41 rotor at 4^{o} C. The numbers at the top of the Figure represent ribosomal RNA standards obtained from *E. coli* (23S and 16S) and calf liver (28S and 18S) and run simultaneously.

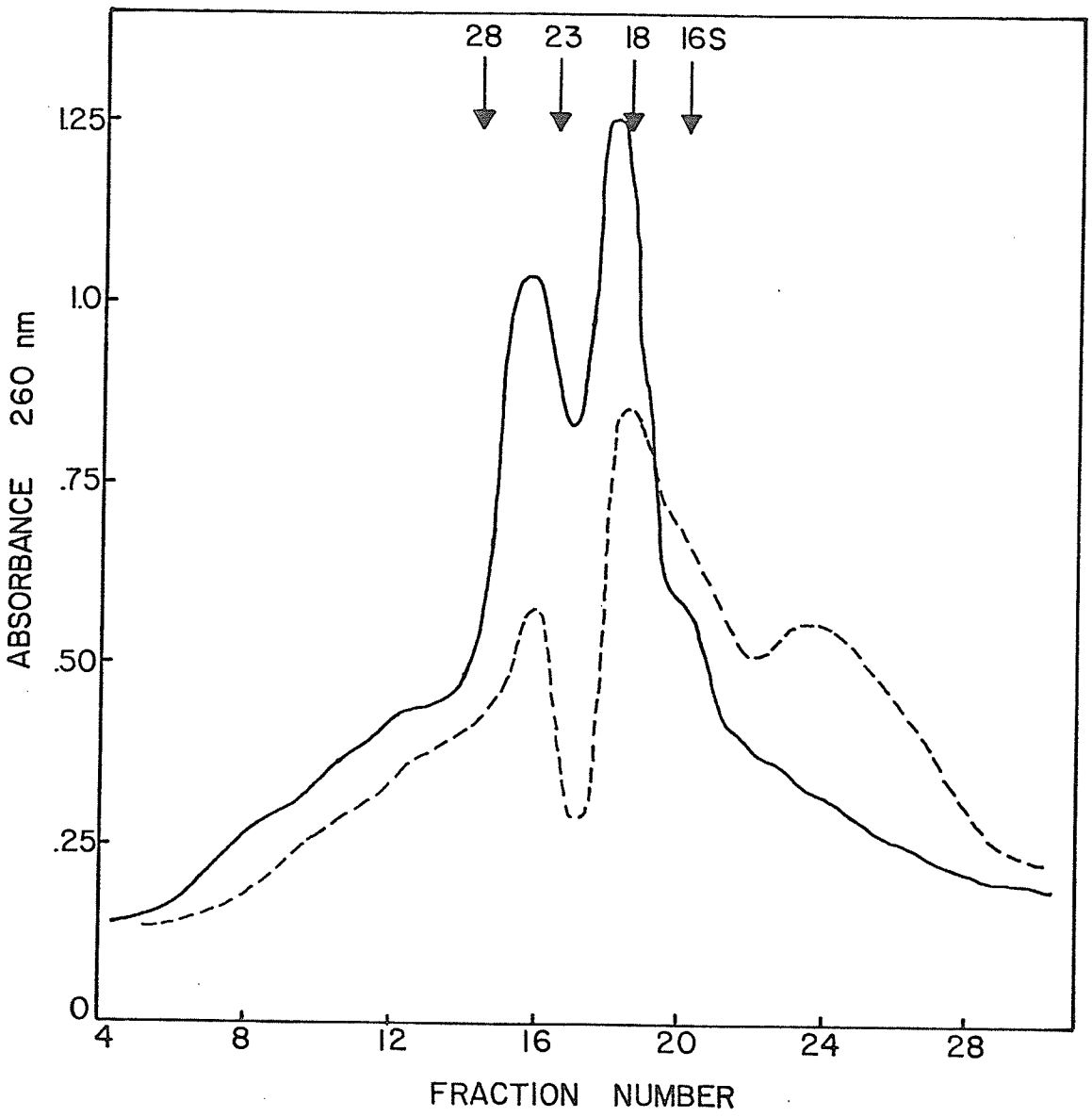

28
23
18
16S
1.25
1.0
.75
.50
.25
0
ABSORBANCE 260 nm
4
8
12
16
20
24
28
FRACTION NUMBER

Figure 41: Characterization of rat liver poly(A)-mRNA by agarose-urea gel electrophoresis.

Electrophoresis was performed on 1.5 % agarose gels containing 6.0 M urea and 0.025 M sodium citrate, pH 3.5 as described in the Experimental Procedures. Tracks 1, 8 and 11 contain standard ribosomal RNA from *E. coli* (23S and 16S), calf liver (28S and 18S) and a mixture of *E. coli* and calf liver standards, respectively. Tracks 2,3, and 4 contain 4.4, 7.3 and 14.5 µg of hepatic poly(A)-mRNA isolated from rats starved for 48 H and refed for 4 H. Tracks 5, 6 and 7 contain 3, 6, and 15 µg of mRNA from rats refed for 4 H with inflammation induced at 0 H of refeeding. Tracks 9 and 10 contain 11.5 and 18 µg of mRNA isolated from free-polysomes from rats refed for 4 H. Figure 41 (A) is an enlargement of Figure 41 (B).

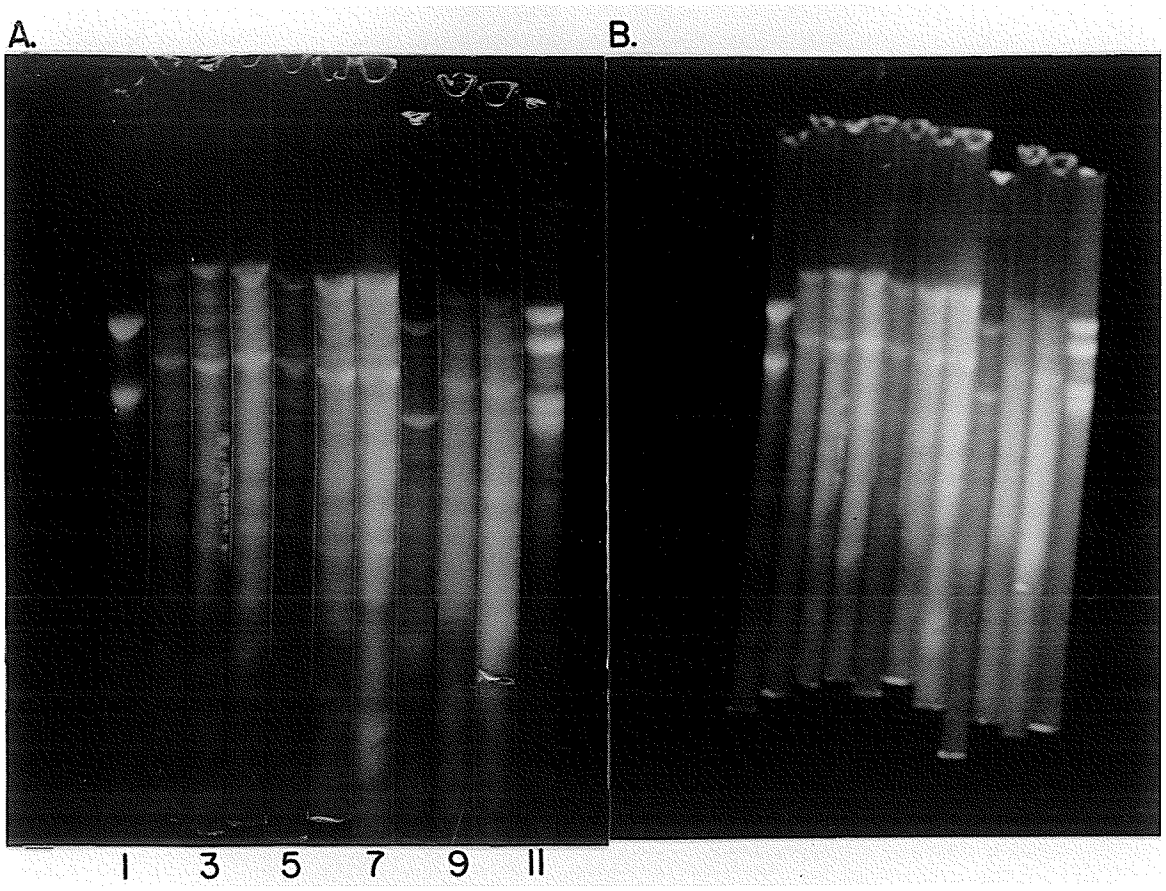
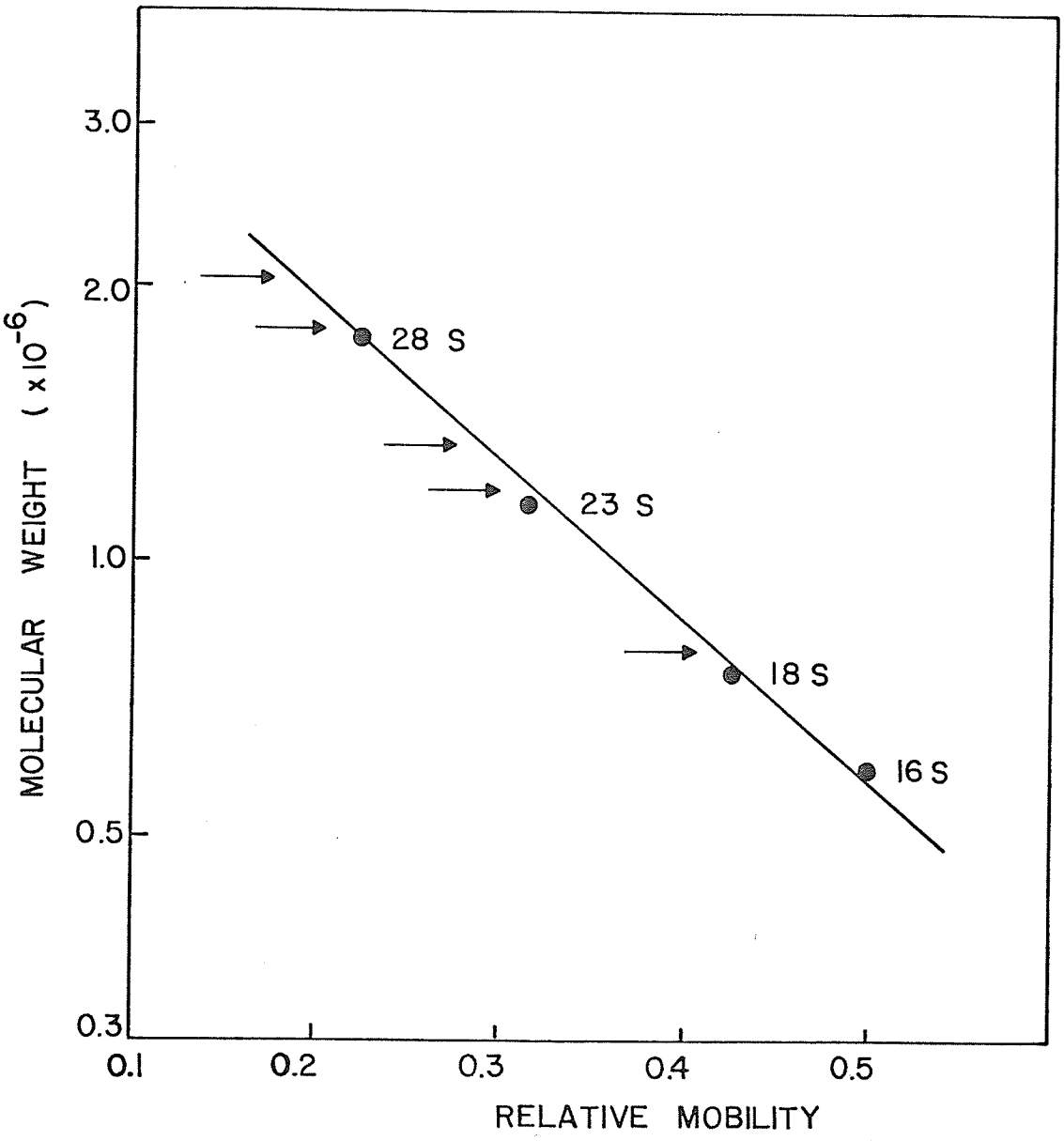

A.
B.
1
3
5
7
9
11

Figure 42: Molecular weight versus relative mobility plot for standard ribosomal RNA.

The gels are shown in Figure 41. The arrows indicate the positions of the migrations of 5 mRNA species visible in Figure 41.


3.0
2.0
1.0
0.5
0.3
MOLECULAR WEIGHT (x10^{-6})
28 S
23 S
18 S
16 S
0.1
0.2
0.3
0.4
0.5
RELATIVE MOBILITY

and indicate the existence of a number of distinct groups of purified mRNA. The approximate size of the largest band (30S or 2.0 x 10^6) Nepokroeff and Porter, 1978; Zehner *et al.*, 1980) is in the range calculated to be required for FAS synthesis.

4.2 Variations in the Levels of Translatable Messenger RNA during Adaptive Synthesis and Inflammation.

The poly(A)-containing messenger RNA was translated in a cell-free, nuclease-treated, rabbit reticulocyte lysate translation system supplied by New England Nuclear, Corp. The methods used in translating the poly(A)-mRNA and identifying the products are described in the Experimental Procedures.

To test the poly(A)-mRNA fractions and to standardize the experimental conditions, a number of characteristics of the lysate system were examined. Figure 43 shows the results of a typical translation where the time of incubation at 37°C was varied. Poly(A)-mRNA was isolated from rats fed a normal diet, rats starved for 48 h and refed a fat-free diet for 12 h and a similar group with inflammation induced at 0 h of refeeding. The total protein counts were determined by measuring the TCA-precipitable counts in an aliquot of each translation mixture. Figures 44A and 44B illustrate the effects of the addition of transfer RNA to the system and variations in the levels of magnesium ions, respectively. The addition of transfer RNA appeared to have little effect upon the translation of either total RNA or specifically the message for FAS. Similarly variations in the magnesium ion concentration from 0.5 mM to 4.0 mM resulted in minimal changes in the levels of translation. The system used in subsequent experiments was as described and differed from that of Lau *et al.*

Figure 43: Time dependence of translation of total poly(A)-mRNA.

All translations involved approximately 1 µg of poly(A)-mRNA from rats fed a normal diet (□——□), starved for 48 H and refed a fat-free diet for 12 H (○——○) and a similar group with inflammation at 0 H (●---●).

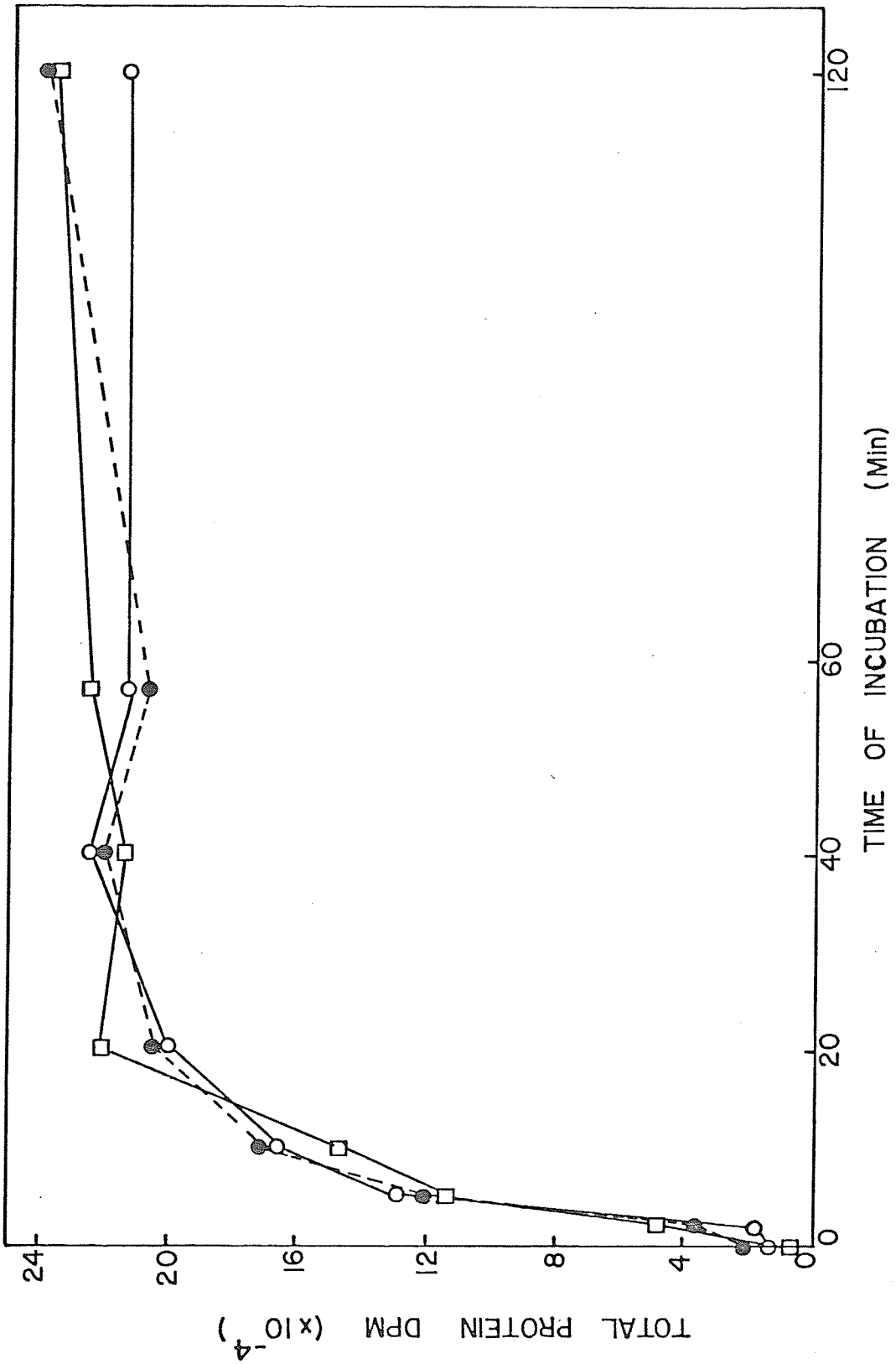
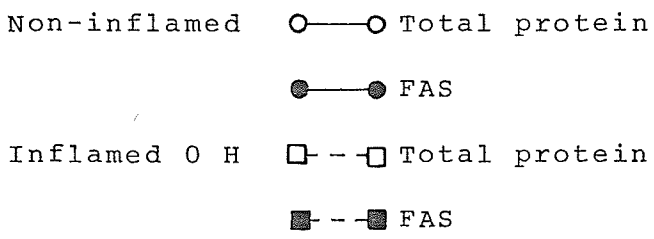
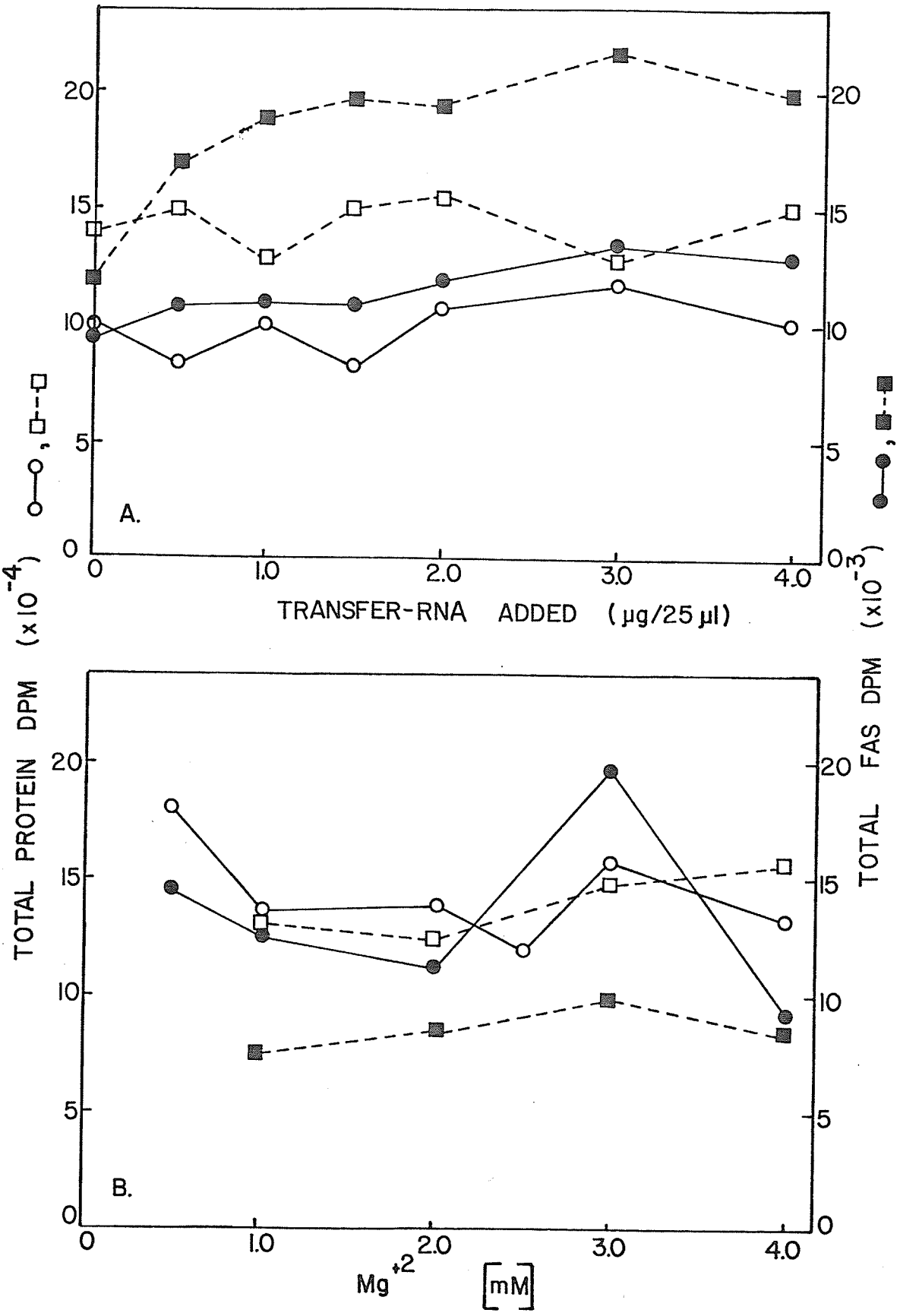

TOTAL PROTEIN DPM (x 10⁻⁴)
0
4
8
12
16
20
24
0
20
40
60
120
TIME OF INCUBATION (Min)

Figure 44: Effects of Transfer RNA and Magnesium levels on the translation of poly(A)-mRNA from rat liver.



A. Effect of varying amounts of Transfer RNA. The poly(A)-mRNA was isolated from rats starved for 48 H and refed a fat-free diet for 12 H with inflammation at 0 H of refeeding. Translation was as described in Experimental Procedures and involved 0.9 µg and 1.2 µg of poly(A)-mRNA from inflamed and non-inflamed animals, respectively.

B. Effect of varying Magnesium levels. The poly(A)-mRNA was isolated from rats starved for 48 H and refed for 24 H with inflammation at 0 H of refeeding. Translation was as described in Experimental Procedures and involved 1.2 µg of poly(A)-mRNA from inflamed and non-inflamed animals, respectively.


A.
TRANSFER-RNA ADDED (µg/25 µl)
(x10⁻⁴)
(x10⁻³)
B.
TOTAL PROTEIN DPM
TOTAL FAS DPM
Mg⁺² [mM]

(1979) who found variations, specifically in the translation of FAS message, by both magnesium and transfer RNA changes. Mattick *et al.* (1981) used 0.5 mM magnesium and 125 µg/ml calf liver tRNA as compared to 1.0 mM magnesium and no tRNA for our system and 2.2 mM and 80 µg/ml for Lau *et al.* (1979).

4.2.1 Variations observed for FAS mRNA from rats starved for 48 h and refed a fat-free diet for 12 h with inflammation at 0 h.

To test the effects of inflammation on the adaptive synthesis of fatty acid synthetase, 12 h of refeeding a fat-free diet and a 0 h inflammation time were chosen (see Table 11). According to the earlier data (Figure 33 and Nepokroeff and Porter, 1978) this is just prior to the largest observed increase in the relative rate of synthesis of rat liver FAS and presumably should show the largest variations in mRNA levels. The effect of varying the amounts of isolated poly(A)-containing mRNA, added to the cell-free translation system is shown in Figure 45. The counts in the total protein synthesized were determined by measuring the label incorporated into the TCA-precipitable fraction of each sample. The counts in FAS were determined by immunoprecipitation as described in the Experimental Procedures. The label in FAS for Figure 45 was determined by counting an aliquot of each immunoprecipitable sample and subtracting the co-precipitation values (as described in the Experimental Procedures). The results for total protein indicate the activity of the total poly(A) message from both inflamed and non-inflamed rats are approximately identical. The results for label incorporated into a protein precipitated by monospecific anti-FAS antiserum indicate a large variation in specific message activity between inflamed and non-inflamed animals. Table 12(A) compares the observed induction of FAS mRNA for non-inflamed and inflamed animals as a

Figure 45: Cell-free synthesis of total protein and immunoprecipitable FAS directed by poly(A)-messenger RNA.

Hepatic poly(A)-containing RNA was isolated as described from rats starved for 48 H and refed a fat-free diet for 12 H. Inflammation was induced at 0 H of refeeding. Total FAS DPM were determined from an aliquot of the immunoprecipitated sample from which the co-precipitation values were subtracted.

Total protein DPM are given by the open symbols (○—○, non-inflamed; □--□, inflamed); while total FAS DPM are given by the closed symbols (●—●, non-inflamed; ■--■, inflamed).

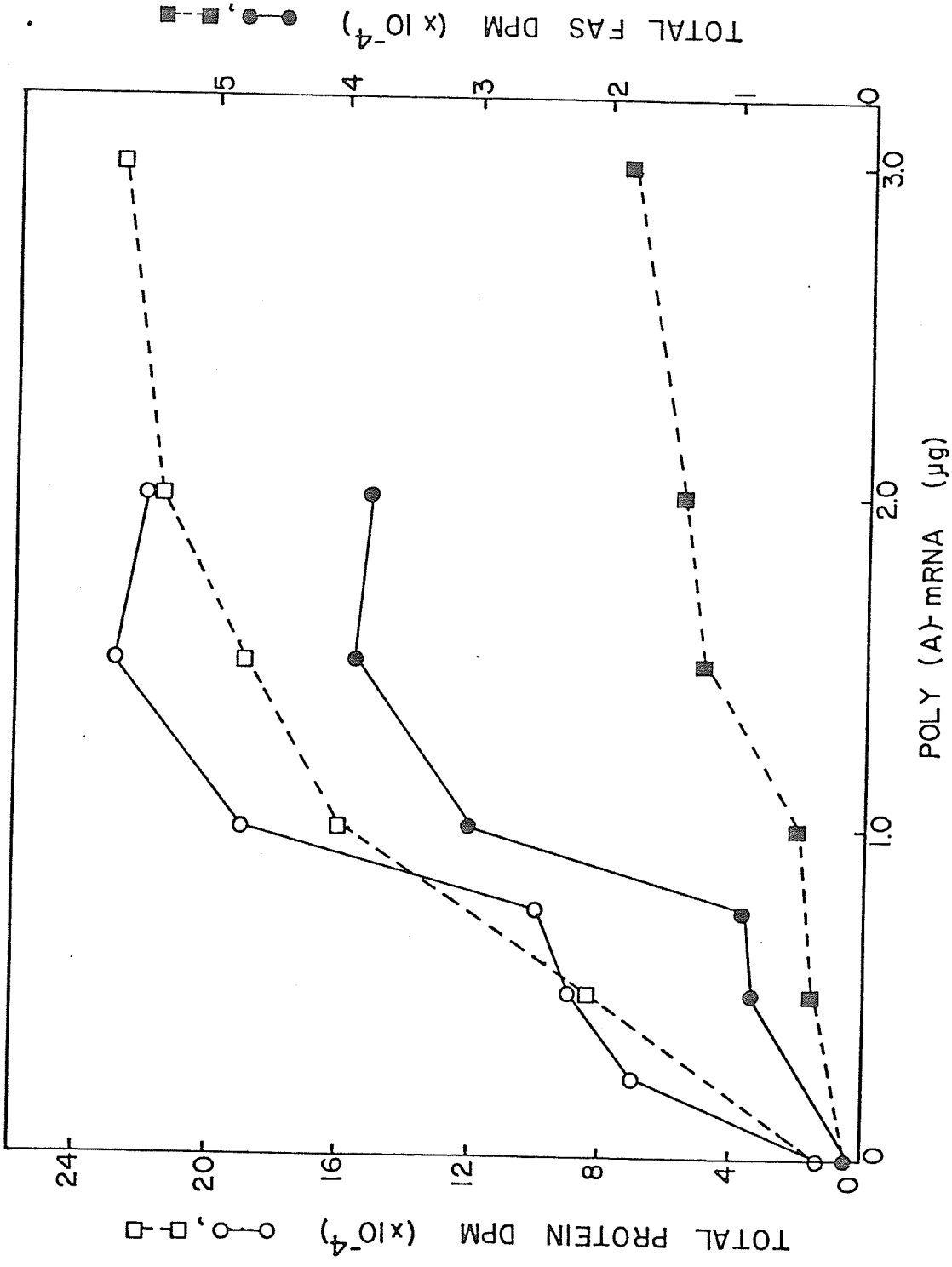

TOTAL PROTEIN DPM (x10⁻⁴) o—o, □—□
TOTAL FAS DPM (x 10⁻⁴) ●—●, ■--■
POLY (A)-mRNA (μg)
0
4
8
12
16
20
24
1
2
3
5
1.0
2.0
3.0

Table 12.

Effect of Adaptive Synthesis and Experimental Inflammation on the Relative Levels of FAS Messenger RNA.

(A)

Messenger RNA added (µg)	$\frac{\text{FAS DPM - Co-Prec. DPM}}{\text{Total Protein DPM}} \times 100$	
	Non-Inflamed	Inflamed
0.25	-	-
0.50	8.9	5.6
0.75	9.9	-
1.0	15.7	3.1
1.5	16.3	5.4
2.0	17	6.8
3.0	-	7.3

(B)

Experimental Description	FAS Levels*	Cross-Reacting Material	Relative Rate of Synthesis	Turnover Index	% FAS mRNA**
Non-Inflamed	250	4.8 mg/liver	10.6	0.596	16.3
Inflamed	125	2.0 "	5.7	1.08	5.4

*FAS levels are given in mM Palmitate Produced/ minute/ liver

**from above Table 12(A).

percentage of the total message. In the range of 1-2 µg of poly(A)-mRNA added to the translation system the FAS mRNA/total protein mRNA ratio averaged 16.3% for the non-inflamed and 5.4% for the inflamed animals. Table 12(B) compares the induction of enzyme activity, cross-reacting material, turnover-index, and the levels of mRNA translating into FAS. Messenger RNA levels for FAS of non-inflamed animals were 3 times those observed for inflamed animals, while the relative rate of synthesis and activity was approximately double. The relative synthesis observed after 24 h of refeeding was 3 times that for inflamed animals perhaps indicating the increased levels of message specific for FAS may require a certain time interval before the differences manifest themselves as synthesized protein in the intact liver cell.

To further identify and characterize the translation products, polyacrylamide gel electrophoresis was performed as described. Analysis of translation products on polyacrylamide gels after immunoprecipitation is a more exacting criterion than measurement of label incorporated after immunoprecipitation alone. Figures 46, 47 and 48 illustrate the electrophoretic analysis of immunoprecipitated translation products. Figure 46 shows the results from 7% polyacrylamide gels (60:1 ratio of acrylamide to bis-acrylamide) for 1 µg of poly(A)-mRNA from non-inflamed and inflamed animals (10 µl sample from 50 µl resuspension of the immunoprecipitated sample was run per gel). Also included is a sample translation of 10 µg of RNA isolated from the high salt wash of the oligo(dT)-cellulose column (i.e. non-poly(A)-containing RNA from the polysomal RNA fraction). Prior to translation the RNA from the high salt wash was precipitated overnight with ethanol and dissolved in distilled water. There was little immunoprecipitated label and what was present may be co-precipitated label or translations

Figure 46: SDS-polyacrylamide gel electrophoresis of purified fatty acid synthetase and the immumoprecipitated labelled protein product synthesized in a rabbit reticulocyte lysate translation system.

The upper portion of the figure is a schematic representation of stained gels upon which a cross-linked hemocyanin standard, purified FAS and an immunoprecipitated translation product were electrophoresed.

Poly(A)-mRNA samples were isolated from rats starved for 48 H and refed a fat-free diet for 12 H (A) and a similar group with inflammation at 0 H of refeeding (B). High salt wash from the oligo(dT)-cellulose column (C) was also translated and run on the 7% gels.

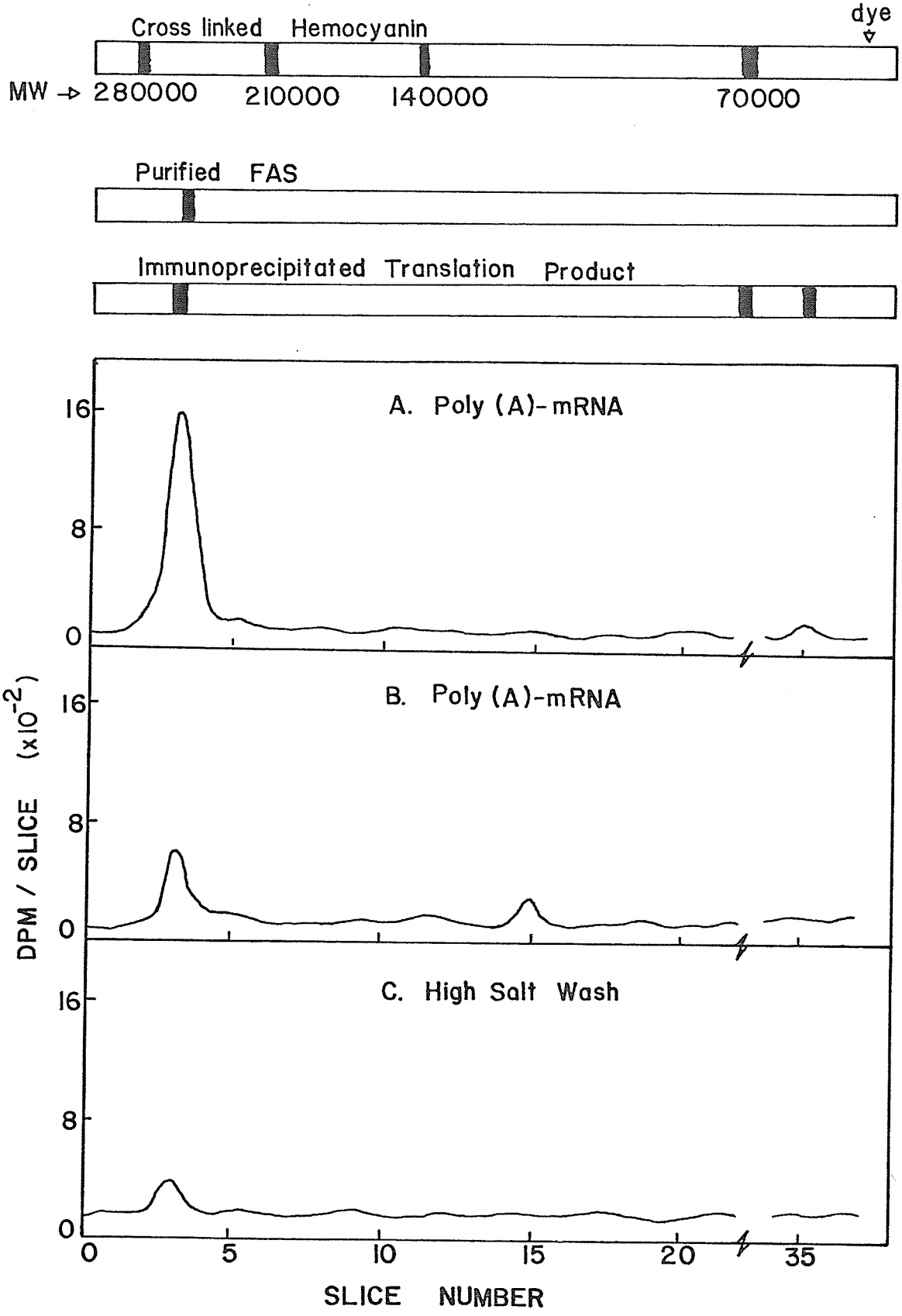

Cross linked
Hemocyanin
dye
MW →
280000
210000
140000
70000
Purified FAS
Immunoprecipitated Translation Product
A. Poly (A)-mRNA
B. Poly (A)-mRNA
C. High Salt Wash
DPM / SLICE (x10^-2)
16
8
0
0
5
10
15
20
35
SLICE NUMBER

Figure 47: Electrophoretic analysis of the immunoprecipitated samples from non-inflamed animals refed for 12 H. Further details are given in the legend for Figure 46.

The numbers (2.0 µg) indicate the number of µg of poly(A)-mRNA added to the translation system. The numbers without arrows indicate the peak attained for that amount of mRNA (data not shown). The letters H and L indicate the position of the heavy and light immunoglobulin chains found with staining.

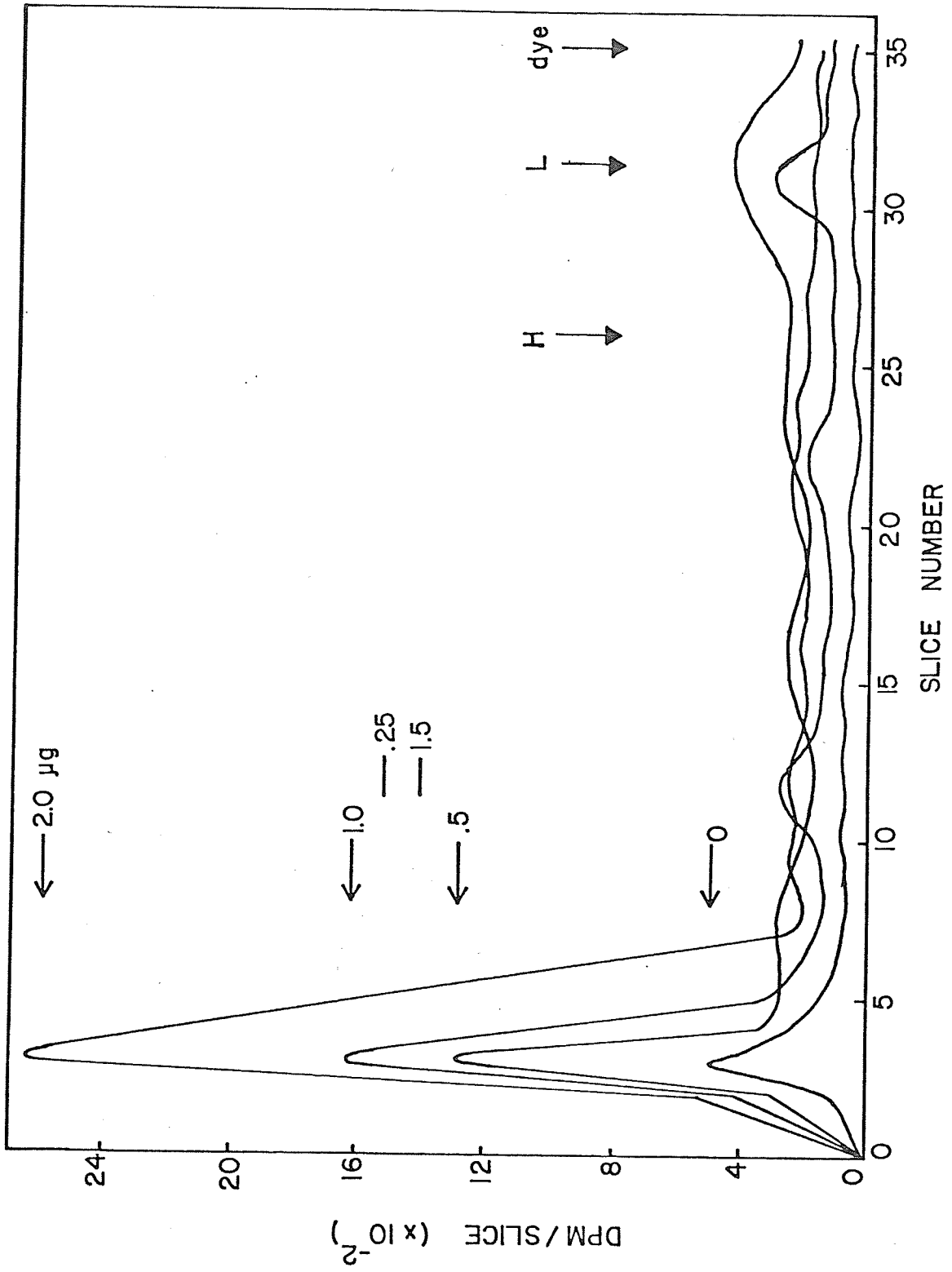
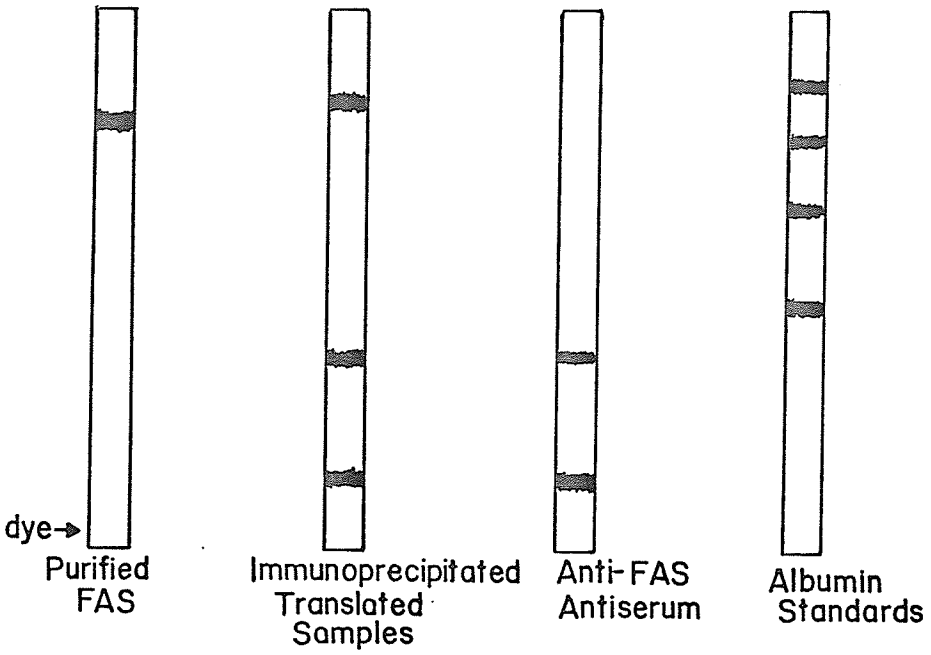
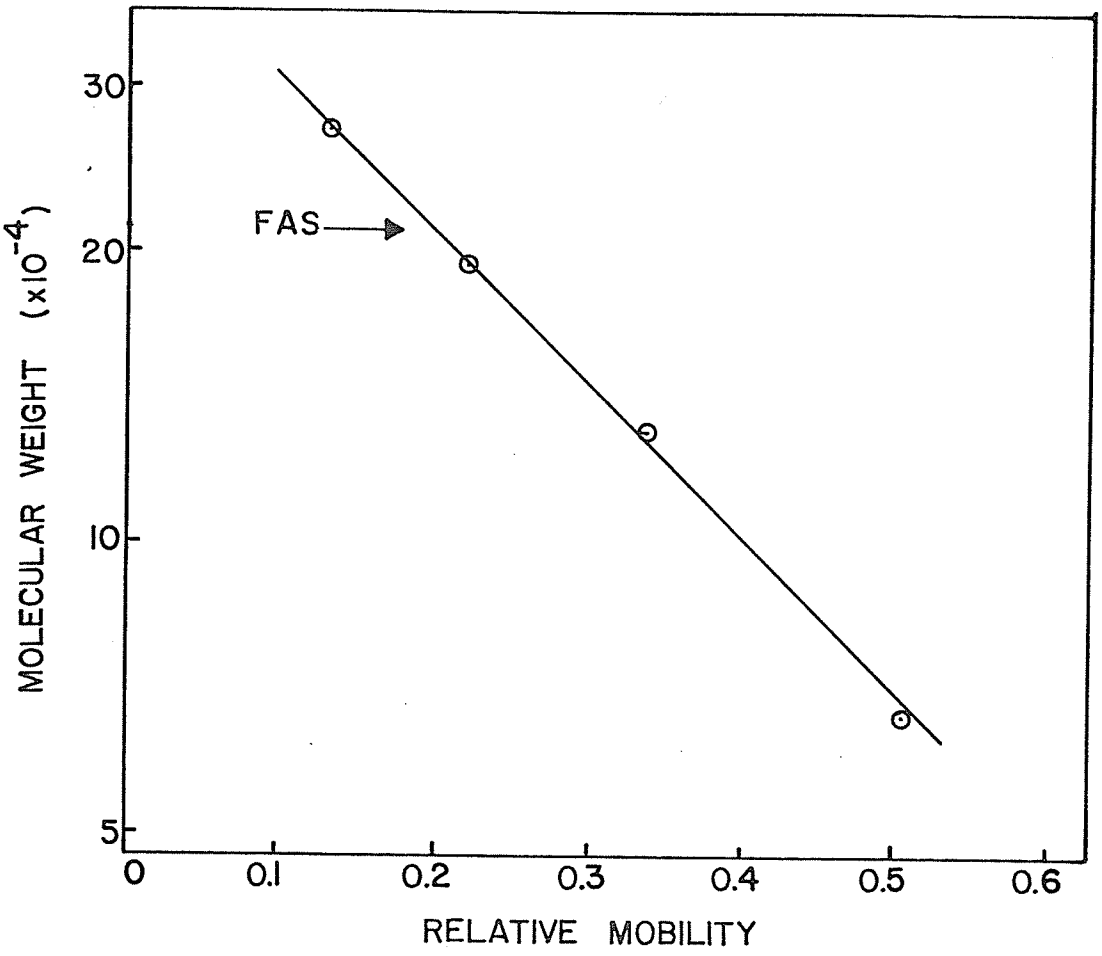

2.0 µg
1.0
.25
1.5
.5
0
H
L
dye
DPM / SLICE (x 10⁻²)
SLICE NUMBER
0
4
8
12
16
20
24
0
5
10
15
20
25
30
35

Figure 48: Electrophoretic analysis of immunoprecipitated translation products.

Aliquots of the translation products which precipitated with anti-FAS antiserum were run on 5% SDS-polyacrylamide gels (30:1 acrylamide to bis-acrylamide ratio) as described in Experimental Procedures. The top of the Figure is a schematic representation of the bands while the bottom plots MW versus relative mobility of the albumin standards and FAS.


dye
Purified FAS
Immunoprecipitated Translated Samples
Anti-FAS Antiserum
Albumin Standards


MOLECULAR WEIGHT (x10^{-4})
30
20
10
5
FAS
0
0.1
0.2
0.3
0.4
0.5
0.6
RELATIVE MOBILITY

from partially degraded FAS messenger RNA. The top of Figure 46 shows a schematic representation of the bands observed on the 7% gels after staining, prior to slicing and counting. A hemocyanin standard and purified FAS sample were run simultaneously and are represented also.

Electrophoretic analysis of the immunoprecipitated samples from the non-inflamed animals is shown in Figure 47. The majority of the label, present on the gel, was located in the same position approximated by purified FAS. Observation of stained gels revealed the existence of smaller molecular weight bands as minor components on some of the gels run. These bands may be fragments related to translated fatty acid synthetase (i.e. partial translation products) or they may be break-down products of the complete FAS molecule such as have been observed previously (see Historical). Electrophoresis of the purified FAS molecule also occasionally produced smaller molecular weight bands depending on the time of storage, etc. Also, in some cases a large amount of label was found associated with the dye front. Sun and Holten (1978) found that radioactivity migrating with the dye front was soluble in 5% trichloroacetic acid suggesting it was non-incorporated labelled amino acids and not fragments of glucose-6-phosphate dehydrogenase which they were investigating. Similar gel electrophoresis was performed on samples from inflamed animals (not shown) and the combined results are presented in Table 13. The values from Table 13 are much less than the values obtained by direct counting of an aliquot of the immunoprecipitated sample (corrected for co-precipitation) but the proportions between inflamed and non-inflamed animals from Table 12A are approximately maintained.

Table 13.

Effect of Adaptive Synthesis and Inflammation on the Relative Levels of Messenger RNA.

Poly(A)-mRNA (µg)	FAS DPM*		% FAS DPM / Total Protein DPM	
	Non-Inflamed	Inflamed**	Non-Inflamed	Inflamed
1.0	8250	3500	4.3	2.2
1.5	7500	3625	3.3	1.9
2.0	13750	3750	6.3	1.7
3.0	-	4000	-	1.7

*Label in FAS was isolated electrophoretically on 7% polyacrylamide gels.

**Rats were starved for 48 H and refed a fat-free diet for 12 H, inflammation was induced after 0 H of refeeding.

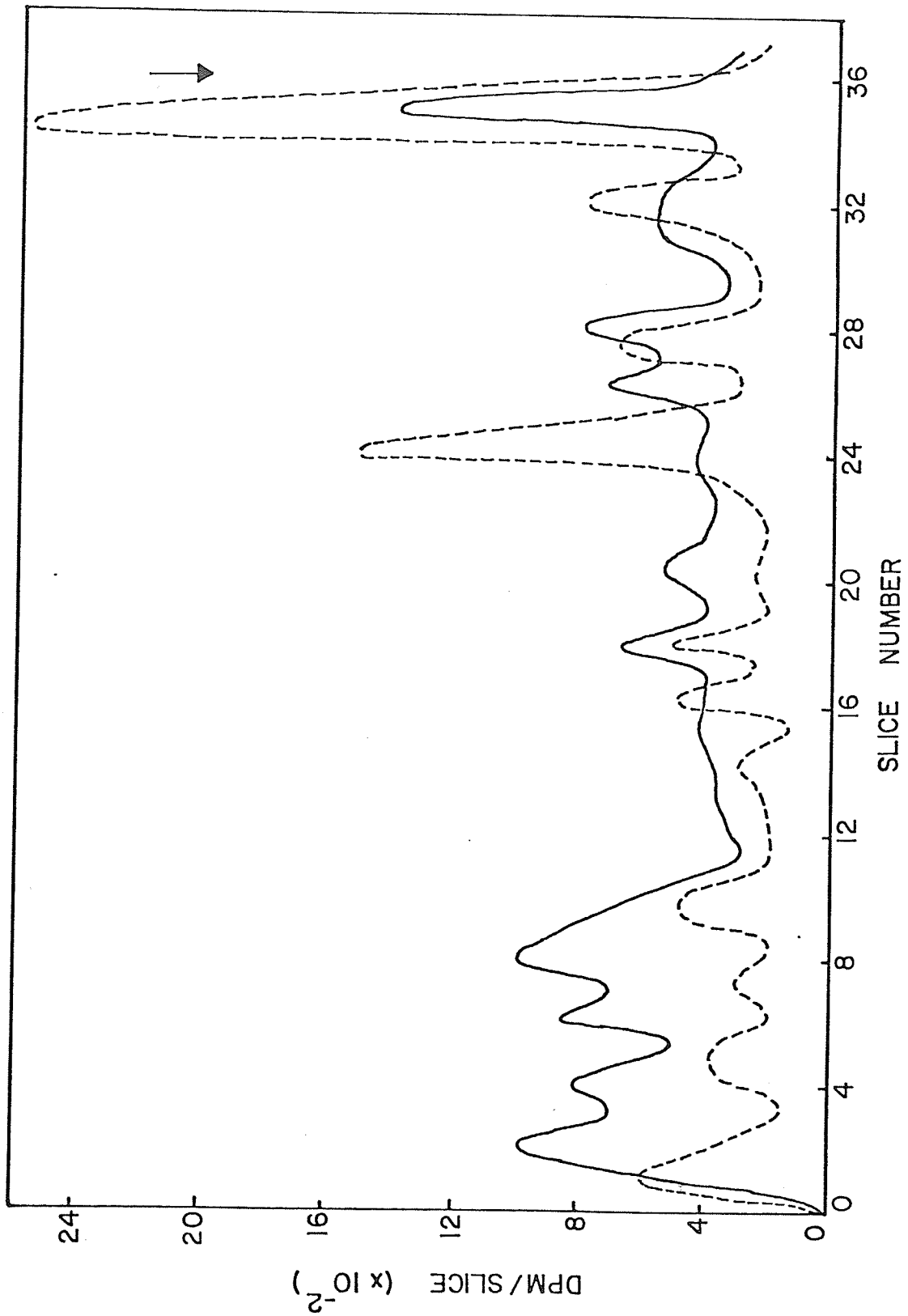
The immunoprecipitated product was also characterized on 5% gels (30:1 acrylamide to bis-acrylamide ratio) as shown in Figure 48. The immunoprecipitated band again migrated in a similar position to that of purified FAS (molecular weight approximately 2.2×10^5). Determination of the radioactivity in the gels yielded similar results to those discussed for 7% electrophoresis.

Figure 49 illustrates the electrophoresis of the total translation products on 5% gels after immunoprecipitation. Approximately one-half the total sample was applied and resulted in observable differences between inflamed and non-inflamed animals. The inflamed animals have less radioactivity remaining in the upper regions of the gel while having an increase in smaller molecular weight proteins. This is in agreement with the earlier observation of an increase in smaller messenger RNA with inflammation, from sucrose density gradient centrifugation. Observation of Figure 49 also indicates a reduction in the expected amount of total radioactivity (i.e. from Figure 45). As in the case of the immunoprecipitated sample the reasons for this are not clear.

The differences observed between inflamed and non-inflamed animals have been presented as per amount of RNA. As shown in Table 11 the amount of RNA per gram wet weight of liver was greater for inflamed (and starved animals) but this is more than compensated for by the increased size of the liver for refed rats without inflammation. From the data presented for rats refed for 12 h it appears that most, if not all, of the difference in the relative synthesis of FAS in inflamed and non-inflamed animals may be accounted for by increased mRNA levels.

Figure 49: Electrophoretic analysis of total translation products on 5% polyacrylamide gels.

One µg of hepatic poly(A)-mRNA, from rats starved for 48 H and refed a fat-free diet for 12 H (solid line) and a similar group with inflammation at 0 H of refeeding (dashed line), was translated, as described, and the products run on 5% gels (after immunoprecipitation). The arrow marks the position of the dye band.


DPM/SLICE (x 10^{-2})
0
4
8
12
16
20
24
SLICE NUMBER
0
4
8
12
16
20
24
28
32
36

4.2.2 Variations in translatable messenger RNA during adaptive synthesis and inflammation.

Shutler *et al.* (1977) showed an increase in the proportion of free-ribosomes with adaptive synthesis and a suppression of this increase by inflammation. More recent work (Nepokroeff *et al.*, 1979) identified the messenger RNA from free-polysomes as being involved in FAS biosynthesis. Table 14 illustrates the translation of poly(A)-mRNA isolated from total polysomal RNA and from free-polysomal RNA. Rats were treated as described in the Table and the poly(A)-mRNA translated in a reticulocyte lysate system. Aliquots of the immunoprecipitated sample were run on polyacrylamide gels and the band corresponding to purified FAS sliced out and counted. The results indicate the majority of counts found in the total polysomal fraction for fatty acid synthetase were also found in the free polysomal mRNA fraction. Table 14 also indicates that inflammation at 0 h of refeeding caused a suppression of FAS mRNA levels at both 4 h and 12 h of refeeding.

Figure 50 shows the effect of refeeding and inflammation upon total protein synthesis by rat liver poly(A)-mRNA. After 48 h starvation the amount of translational activity per µg of mRNA appears to be approximately ½ that found after 12 or 24 h of refeeding. This increase in total mRNA activity for both inflamed and non-inflamed animals appeared in 3 experiments. It may be an artifact of some form of differential isolation or may involve some form of messenger RNA activation. Comparison of the total protein synthesis per liver showed an even larger difference between starved and refed rats, due to the much larger liver size after refeeding.

Figure 51 compares the levels of FAS poly(A)-mRNA for rats refed a fat-free diet for 0, 12 and 24 h with inflammation at 0 h of refeeding.

Table 14.

Translation of Hepatic Poly(A)-mRNA Isolated from Total Polysomes and Free-Polysomes.

Duration of fat-free feeding (H).[a]	Time of inflammation[b]	Polysomal Poly(A)-mRNA (DPM/µg mRNA) Protein	FAS	%[c]	Free-Polysomal Poly(A)-mRNA (DPM/µg mRNA) Protein	FAS	%[c]
4		100,000	4100	4.2	95,000	5225	5.5
4	0	85,000	2125	2.2	104,000	2288	2.2
12		125,000	6125	4.9	140,000	4620	3.3
12	0	120,000	2280	1.9	165,000	3300	2.0

[a] Rats were starved for 48 H prior to refeeding a fat-free diet.

[b] Inflammation was induced by a subcutaneous injection of oil of turpentine at 0 H of refeeding.

[c] $\% = \frac{\text{FAS DPM/µg mRNA}}{\text{Protein DPM/µg mRNA}} \times 100$

Figure 50: Effect of adaptive synthesis and inflammation upon total protein synthesis directed by rat liver poly(A)-mRNA.

Rats were starved for 48 H and refed a fat-free diet for various times as indicated. Inflammation was induced at 0 H of refeeding. Poly(A)-mRNA was isolated and translated as described in the Experimental Procedures. Each point represents as average value obtained using 2 rats. Data for inflamed animals are indicated by solid symbols, while open symbols represent non-inflamed animals.

Figure 50(A) illustrates the total TCA precipitable counts obtained per liver while Figure 50(B) illustrates the counts per µg of poly(A)-mRNA. The level for rats maintained on a normal diet is given by N.D.———. Individual values varied from this average by less than ± 15%.

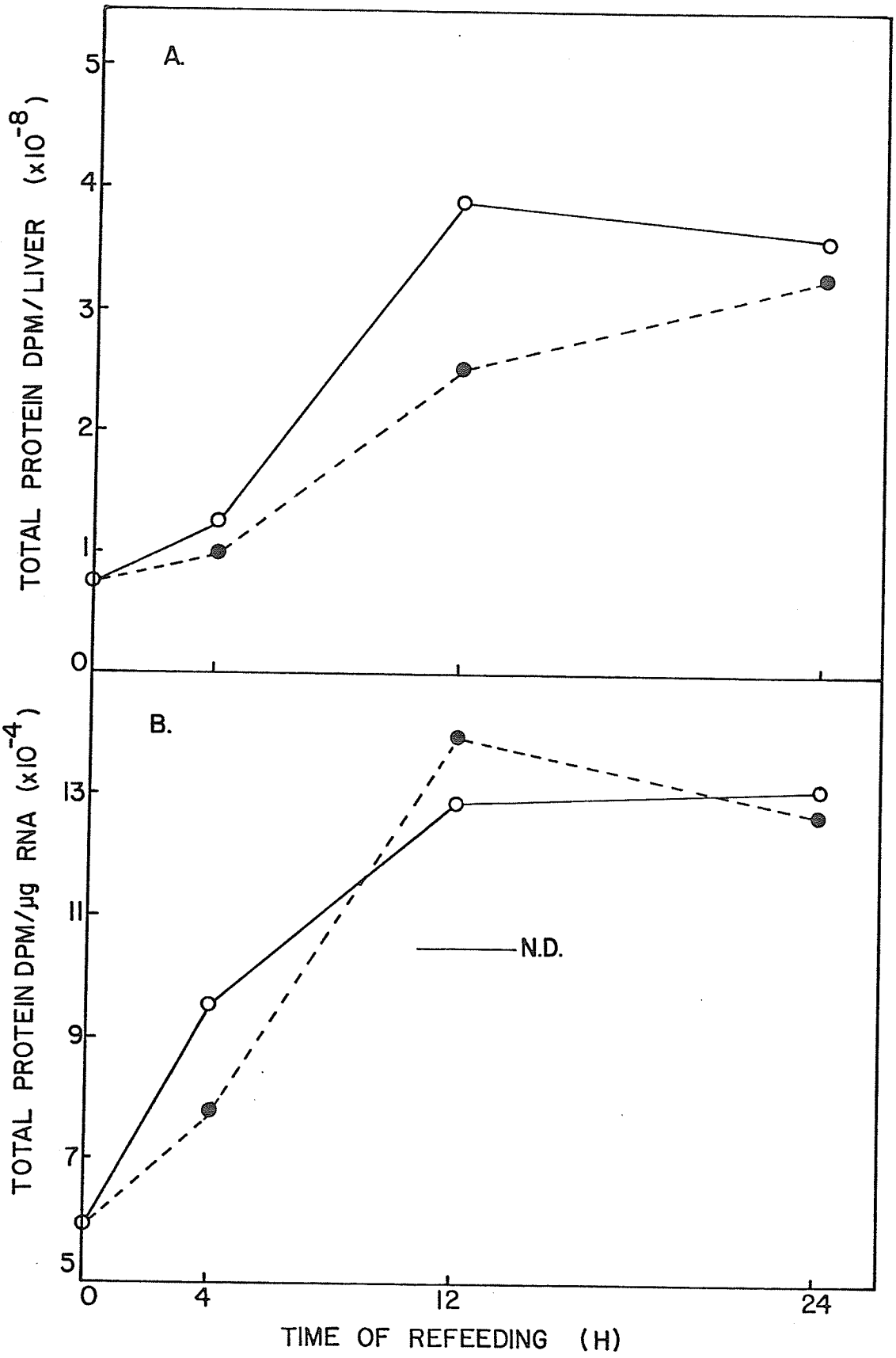
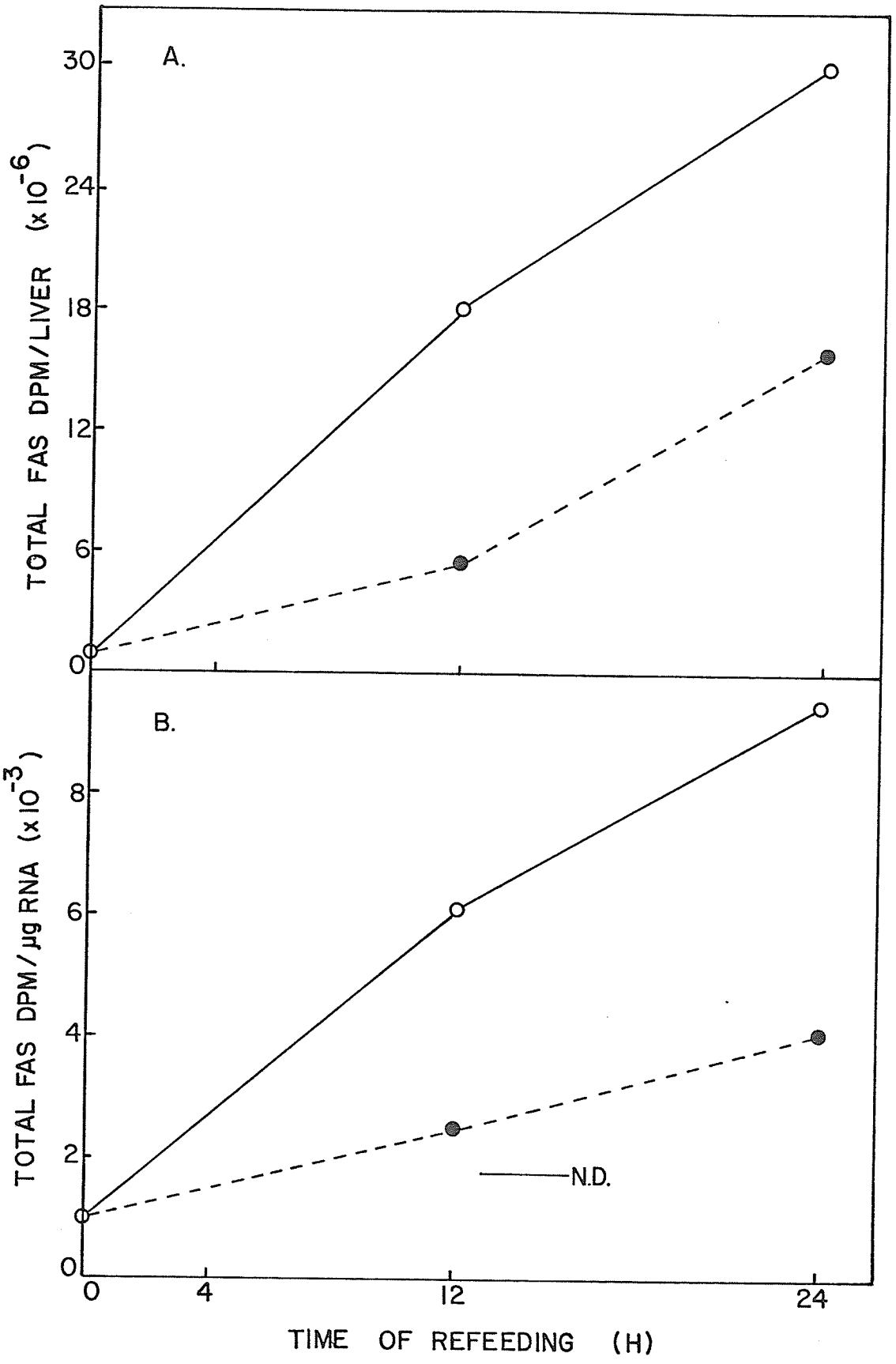

A.
B.
TOTAL PROTEIN DPM/LIVER (x10⁻⁸)
TOTAL PROTEIN DPM/µg RNA (x10⁻⁴)
N.D.
TIME OF REFEEDING (H)
0
4
12
24
5
4
3
2
1
0
13
11
9
7
5

Figure 51: Effect of adaptive synthesis and inflammation upon the synthesis of FAS directed by rat liver poly(A)-mRNA.

Further details are given in the legend to Figure 50. The incorporated label (into FAS) was isolated electrophoretically as described.


A.
B.
TOTAL FAS DPM/LIVER (x10⁻⁶)
TOTAL FAS DPM/µg RNA (x10⁻³)
30
24
18
12
6
0
8
6
4
2
0
N.D.
0
4
12
24
TIME OF REFEEDING (H)

The incorporated label was isolated by electrophoresis of the immunoprecipitate as described previously. Adaptive synthesis was found to increase the levels of FAS DPM per liver after 24 h of refeeding by 30-fold over the starved animals. This increase is suppressed by 50% by a 0 h injection of turpentine.

Table 15 summarizes the data from Figures 50 and 51 and indicates that adaptive synthesis increased the % FAS mRNA/Total Protein mRNA from 1.6 to 7.5% in 24 h. Inflammation suppressed this increase by 60%. The levels of poly(A)-mRNA are given in Figures 50 and 51 and compared in Table 15. Comparison of these results with earlier data (Fig. 11 and Fig. 33) indicates that the activity of FAS rose 25-fold after starvation and the relative rate of synthesis rose 12-fold. The 5-fold increase in the FAS mRNA/Total protein mRNA ratio cannot fully account for the increased synthesis unless translational mechanisms are proposed (see discussion). Another possibility is that the total protein mRNA level for starved animals is artifically low.

Table 15.

Comparison of the Relative FAS mRNA Levels during Adaptive Synthesis and Inflammation.

Duration of fat-free feeding (H).[a]	% Total FAS mRNA / Total Protein mRNA	
	Non-Inflamed	Inflamed[b]
0	1.6	-
4[c]	4.2	2.2
12	4.2	1.8
24	7.5	3.2
Normal Diet	1.7	

[a] Rats were starved for 48 H prior to refeeding a fat-free diet.

[b] Inflammation was induced at 0 H of refeeding.

[c] Taken from Table 14.

5. HORMONAL CHANGES DURING THE SUPPRESSION OF ADAPTIVE SYNTHESIS BY EXPERIMENTAL INFLAMMATION.

As the previous sections have shown, the influence of inflammation on adaptive synthesis, and the process of adaptive synthesis itself, involve complex cellular mechanisms of synthesis and degradation. The changes in synthesis appear to be dependent upon changes in the levels of translatable message although a more detailed study involving complementary-DNA probes is required to elucidate the mechanisms of transcriptional changes.

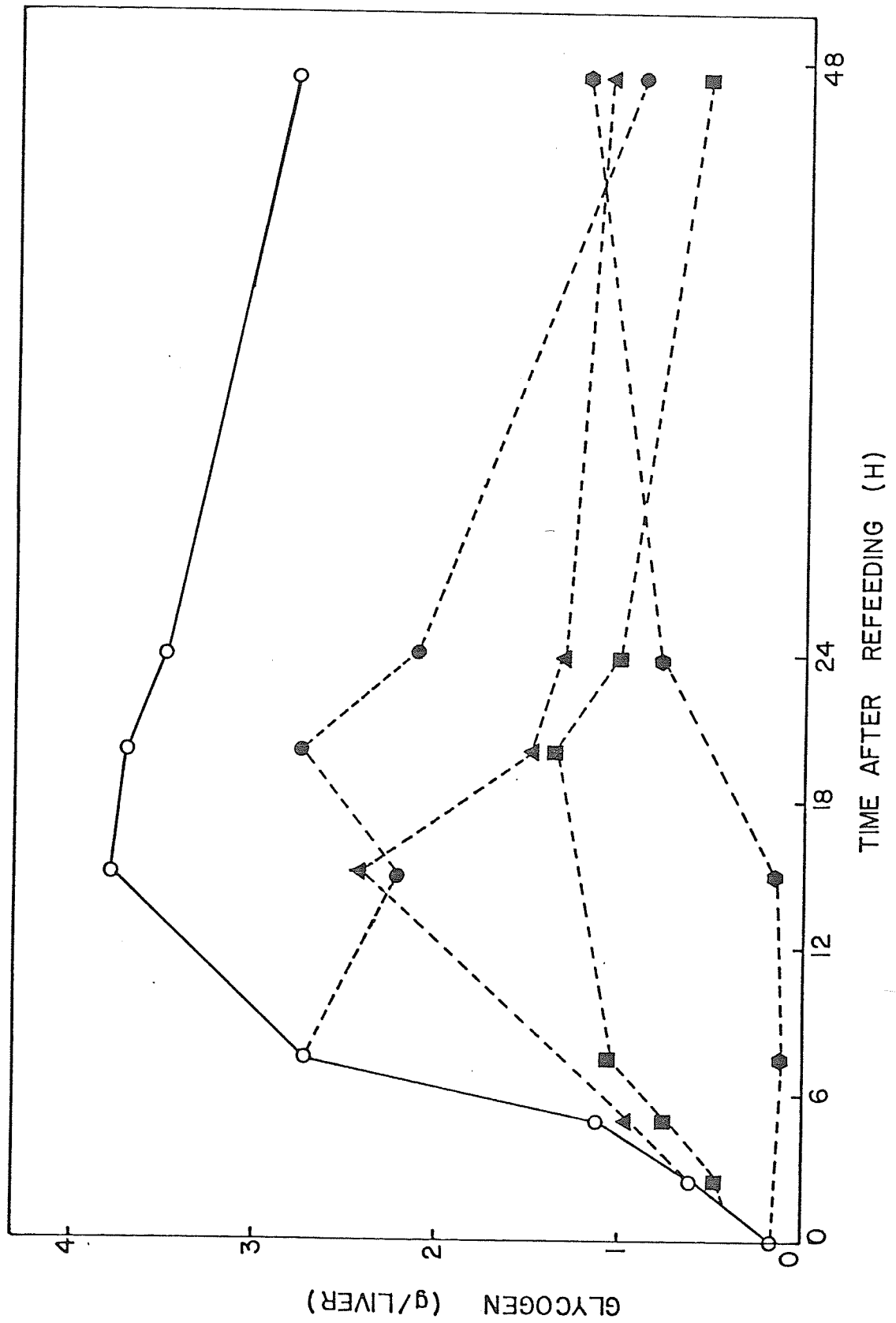
In an attempt to correlate the observed variations during adaptive synthesis and inflammation with various hormonal effects and conditions a number of studies were undertaken. The changes in the hepatic levels of glycogen and cyclic-AMP; and in the serum levels of insulin, cortisol and glucose were investigated in rats starved for 48 h and refed a fat-free diet for up to 48 h. Experimental inflammation was induced at various times 1 h after the start of refeeding by a subcutaneous injection of oil of turpentine.

5.1 Effects of Adaptive Synthesis and Inflammation on the Hepatic Levels of Glycogen.

Liver glycogen levels are intimately related to blood sugar levels (Hers, 1976), and as adaptive synthesis is a result of feeding a fat-free diet containing 68% sucrose, an elevation of liver glycogen during adaptive synthesis was expected. Figure 52 shows that there was a greater than 10-fold increase in the level of liver glycogen after 24 h of refeeding a fat-free diet. The level of liver glycogen decreases slightly with a further 24 h of refeeding. The figure also shows that turpentine injections at various times during adaptive synthesis cause a rapid drop in the liver glycogen level. The same effect is observed

Figure 52: Effects of fat-free refeeding and experimental inflammation on the glycogen content of rat liver.

Rats were starved for 48 H prior to feeding a fat-free diet for up to 48 H. Inflammation was induced at 0 H (●--●), 1.5 H (■--■), 2.5 H (▲--▲) and 7.5 H (●--●) after the start of refeeding. The open circles represent the data for non-inflamed animals. Liver glycogen was measured as described in the Experimental Procedures.


GLYCOGEN (g/LIVER)
0
1
2
3
4
0
6
12
18
24
48
TIME AFTER REFEEDING (H)

when inflammation was induced in rats fed a normal diet, indicating that the effect of inflammation on the glycogen content of liver is independent of adaptive synthesis of fatty acid synthetase.

5.2 Effects of Adaptive Synthesis and Inflammation on the Hepatic Levels of Cyclic-AMP.

Figure 53 shows the effect of inflammation on cAMP levels in rats fed a normal diet. Figure 54 shows cAMP levels in livers of rats in which inflammation was induced 1½, 2½ (Fig. 54A), 5 or 10 h (Fig. 54B) after the start of refeeding. The cAMP levels declined markedly under conditions when adaptive synthesis of FAS was proceeding unhampered by inflammation. This decline was arrested and reversed by the induction of inflammation, particularly when it occurred relatively soon after the start of refeeding.

5.3 Effects of Adaptive Synthesis and Inflammation on the Serum Levels of Glucose, Insulin and Cortisol.

Starvation for 48 h followed by feeding a fat-free diet, caused a rapid rise in serum glucose levels within 12 h of the onset of refeeding (Fig. 55). When inflammation was induced 6 or 8 h after the start of refeeding, the serum glucose levels increased further, and remained relatively high compared to the level in non-inflamed animals (Fig. 55), indicating that inflammation interfered with the regulation of glucose levels. The inset in Figure 55 shows that inflammation alone, uncomplicated by adaptive synthesis, caused a rapid temporary increase in serum glucose levels. This effect is probably related to the interaction between inflammation and glycogenolysis.

As shown in Figure 56, fat-free feeding following starvation did not cause an appreciable increase in serum insulin levels until about

Figure 53: Effect of inflammation on hepatic cAMP levels in rats fed a normal diet.

Rats were maintained on a normal diet during the experiment and cAMP levels measured as described.

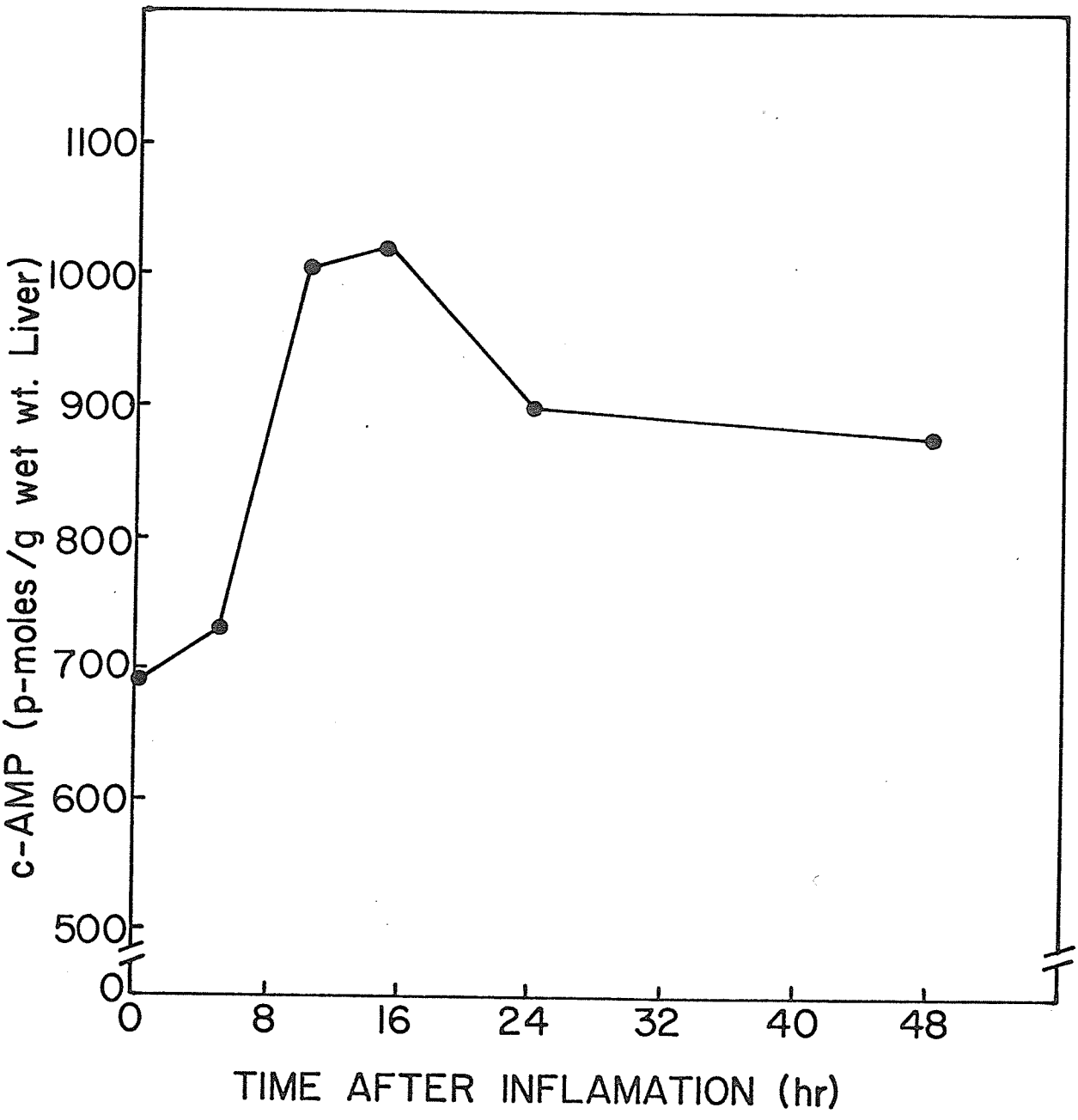

1100
1000
900
800
700
600
500
0
c-AMP (p-moles/g wet wt. Liver)
0
8
16
24
32
40
48
TIME AFTER INFLAMATION (hr)

Figure 54: Effects of inflammation and refeeding on hepatic cAMP levels.

Rats were starved 48 H before feeding a fat-free diet. Inflammation was induced at 1.5 and 2.5 (A), and 5 and 10 H (B) after refeeding (arrows). The animals were killed at the times indicated and liver cAMP was measured as described in Experimental Procedures. Control, without inflammation, ○—○; inflamed 1.5 H after refeeding ▲—▲; inflamed 2.5 H after refeeding ■—■; inflamed 5 H after refeeding ⬢—⬢: and inflamed 10 H after refeeding ◆—◆. Each point represents an average value obtained using 2-3 rats.

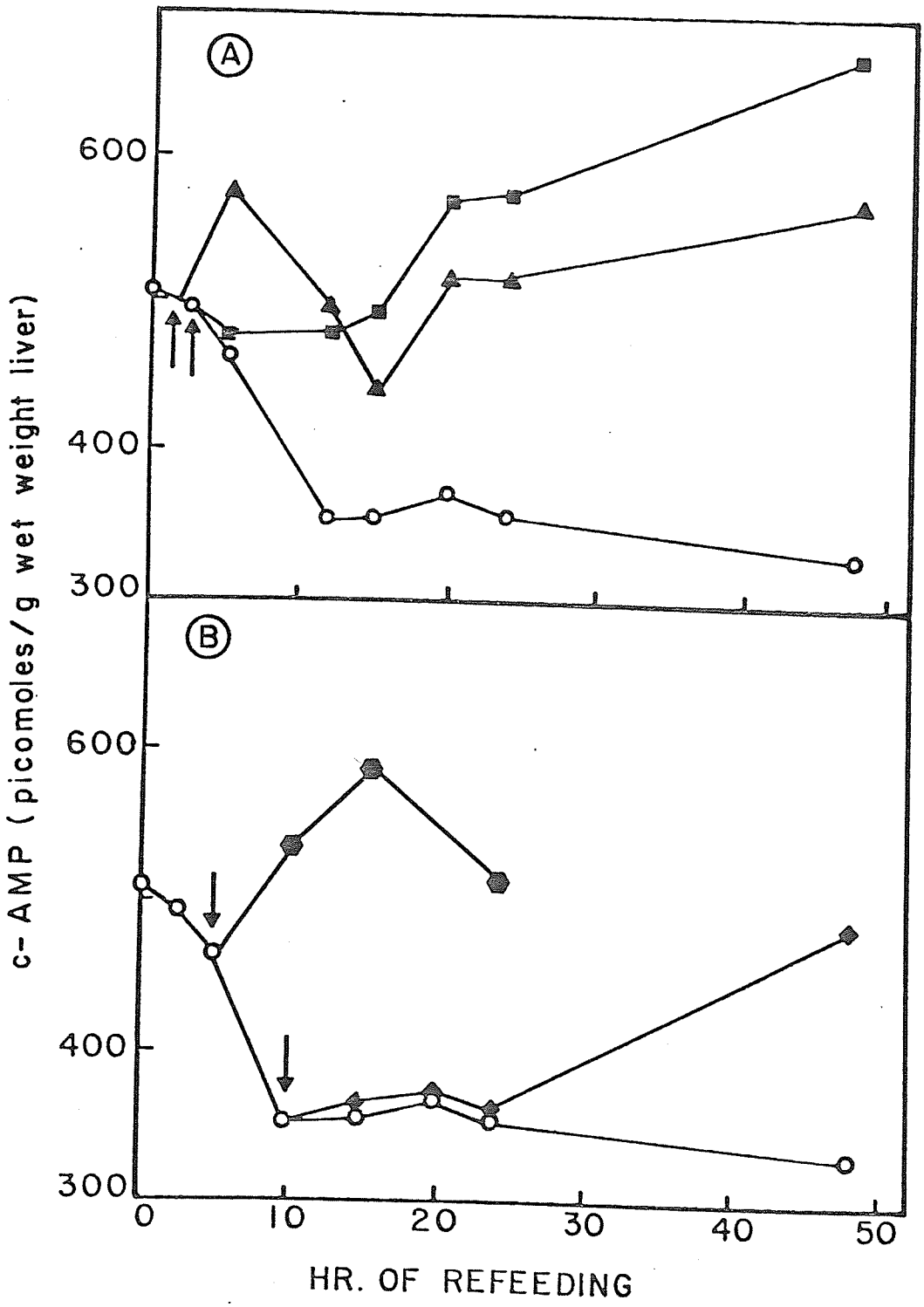

A
B
600
400
300
600
400
300
0
10
20
30
40
50
c-AMP (picomoles/g wet weight liver)
HR. OF REFEEDING

Figure 55: Effects of refeeding and inflammation on serum glucose levels.

Rats were starved 48 H before refeeding a fat-free diet. Inflammation was induced 6 H and 8 H after refeeding (arrows). Serum glucose was determined as described in Experimental Procedures after animals were killed at the times indicated. Control, without inflammation, ●—●; inflamed 6 H after refeeding ⊡--⊡; inflamed 8 H after refeeding △--△. The inset shows the effect of inflammation on animals fed normal lab chow rather than a fat-free diet. Rats were killed at the indicated times after inflammation, and were starved for 16 H before killing. Each point represents an average value obtained using 2-5 rats.

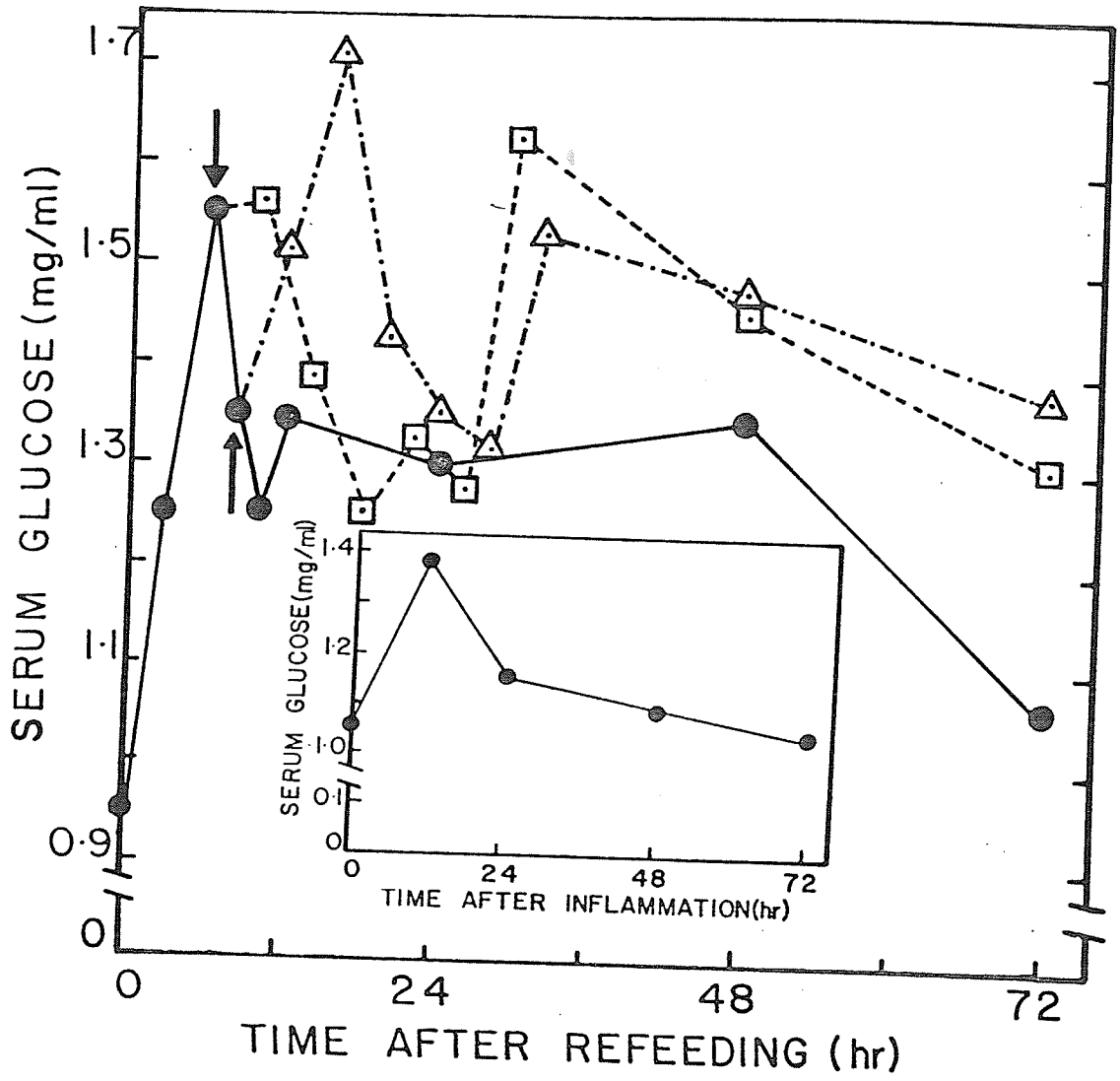
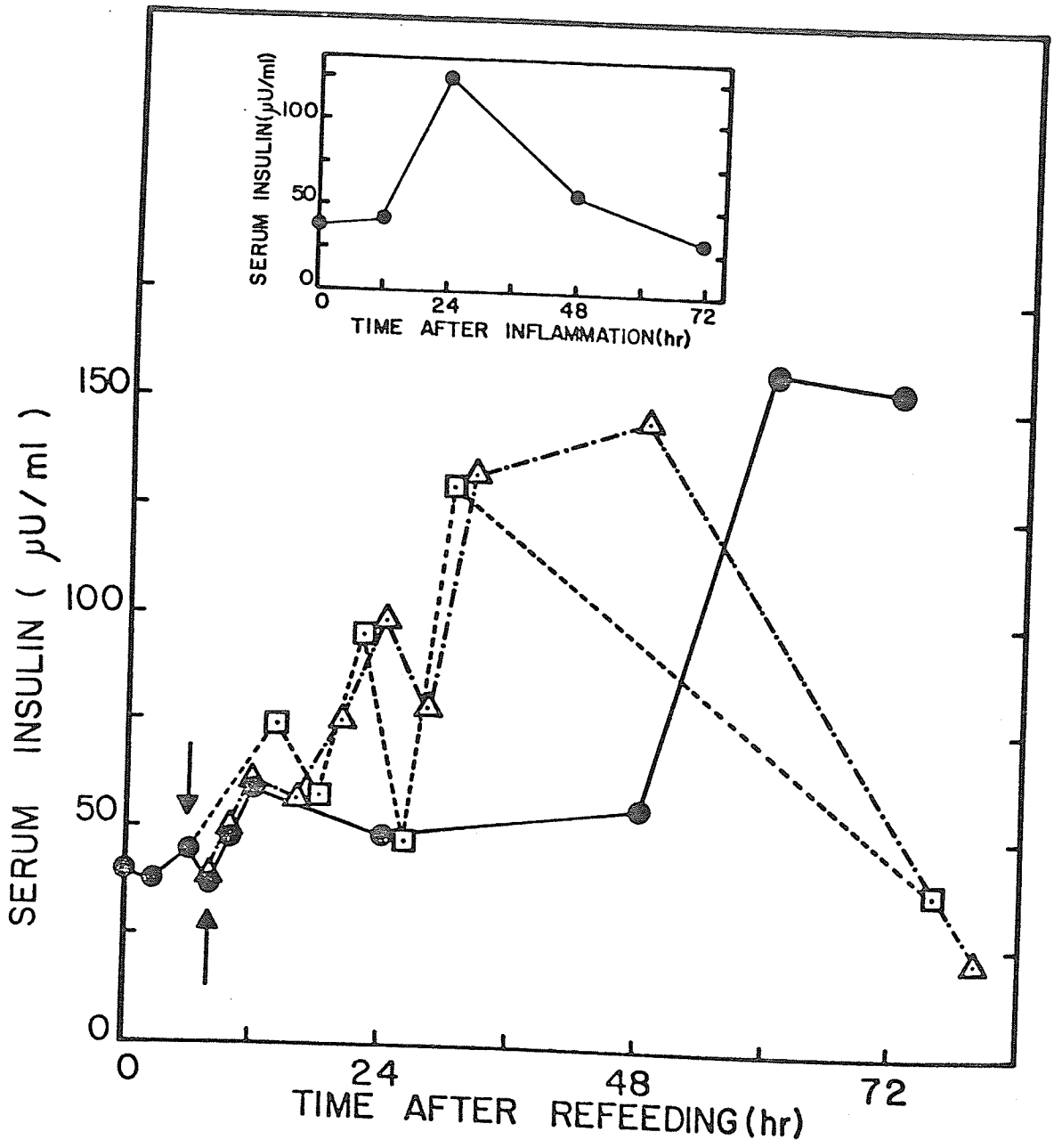

SERUM GLUCOSE (mg/ml)
1·7
1·5
1·3
1·1
0·9
0
0
24
48
72
TIME AFTER REFEEDING (hr)
SERUM GLUCOSE(mg/ml)
1·4
1·2
1·0
0·1
0
0
24
48
72
TIME AFTER INFLAMMATION(hr)

Figure 56: Effects of refeeding and inflammation on serum insulin levels.

Rats were starved 48 H before feeding a fat-free diet. Inflammation was induced 6 H and 8 H after refeeding (arrows). Serum insulin was measured, as described in Experimental Procedures, after animals were killed at the times indicated. Control without inflammation, ●——●; inflamed 6 H after refeeding, ⊡--⊡; inflamed 8 H after refeeding, △--△. The inset shows the effect of inflammation on animals fed normal lab chow rather than a fat-free diet. Rats were killed at the indicated times after inflammation, and were starved 16 H before killing. Each point represents an average value obtained using 2-5 rats.

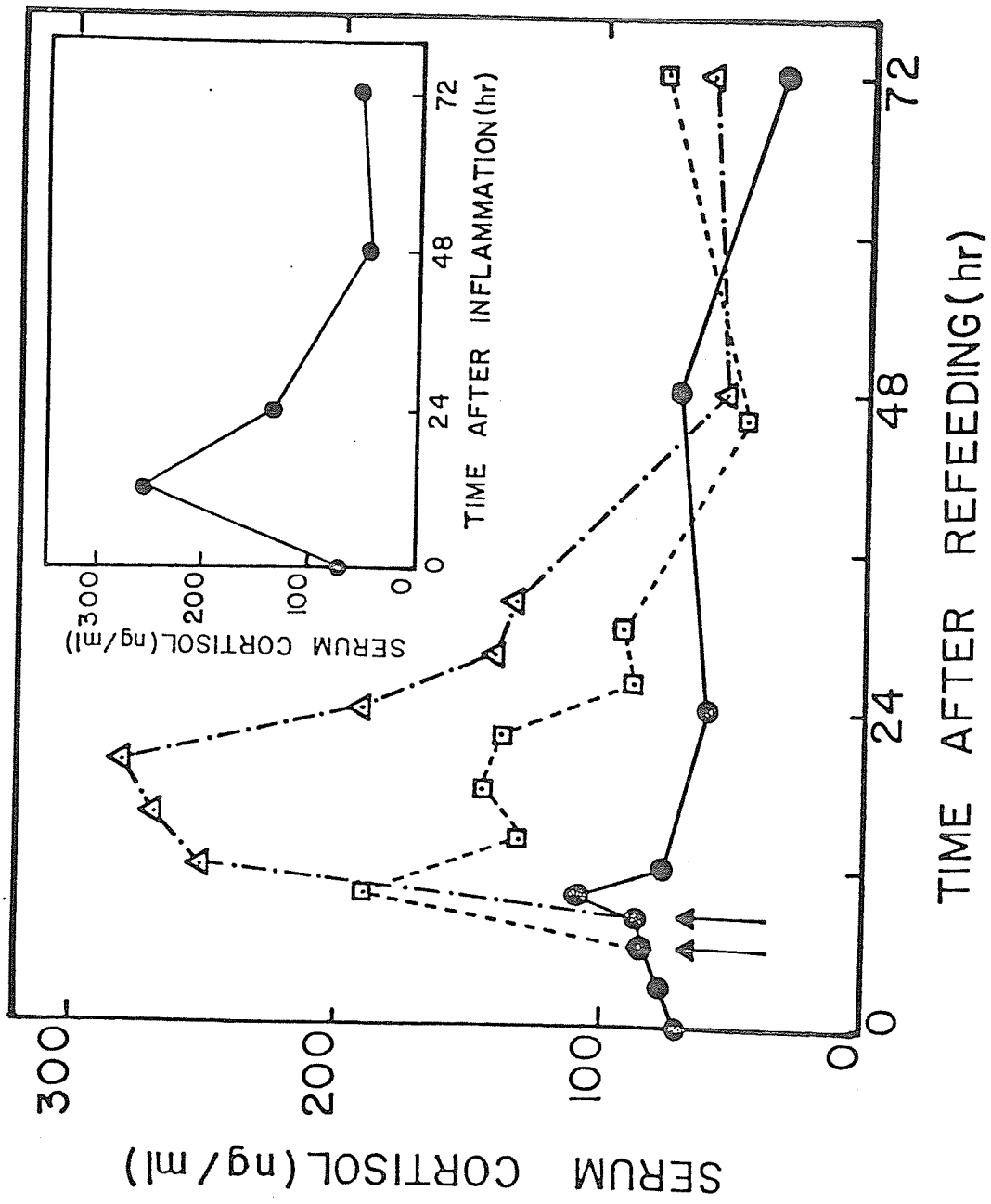

SERUM INSULIN(μU/ml)
100
50
0
0
24
48
72
TIME AFTER INFLAMMATION(hr)
SERUM INSULIN (μU / ml)
150
100
50
0
0
24
48
72
TIME AFTER REFEEDING (hr)

48 h after the onset of refeeding, despite the fact that serum glucose levels were markedly elevated under the same conditions. Inflammation, either alone (inset in Fig. 56) or when induced after starvation and refeeding, caused an elevation in serum insulin levels. This increase with inflammation was delayed about 12 h compared to the increased glucose levels observed under the same conditions.

Figure 57 shows that fat-free feeding following starvation has little, if any, effect on serum cortisol levels. Inflammation induced 6 or 8 h after the onset of refeeding caused a marked elevation in serum cortisol which persisted for 24 h. Inflammation alone, without fat-free feeding, had a similar effect (inset, Fig. 57).

Figure 57: Effects of refeeding and inflammation on serum cortisol levels.

Rats were starved 48 H before refeeding a fat-free diet. Inflammation was induced 6 H and 8 H after refeeding (arrows). Serum cortisol was measured, as described in Experimental Procedures, after animals were killed at the times indicated. Controls, without inflammation, ●——●; inflamed 6 H after refeeding, ⊡--⊡; inflamed 8 H after refeeding, △--△. The inset shows the effect of inflammation on animals fed normal lab chow rather than a fat-free diet. Rats were killed at the indicated times after inflammation, and were starved for 16 H before killing. Each point represents an average value obtained using 2-5 rats.


SERUM CORTISOL (ng/ml)
300
200
100
0
0
24
48
72
TIME AFTER REFEEDING (hr)
SERUM CORTISOL (ng/ml)
300
200
100
0
0
24
48
72
TIME AFTER INFLAMMATION (hr)

6. EFFECTS OF ADAPTIVE SYNTHESIS AND EXPERIMENTAL INFLAMMATION ON OTHER ASPECTS OF LIPOGENESIS.

A preliminary investigation into the effects of starvation, refeeding a fat-free diet and inflammation upon some other aspects of lipogenesis was performed. Starvation and adaptive synthesis are known to have generalized effects causing widespread changes in the cellular distribution of various enzymes. Inflammation is known to increase the extent of cellular membranes (see Historical), the lipid components of which are produced via an alternate route from acetyl-CoA than the fatty acid biosynthetic pathway. Recent (Coolbear and Mookerjea, 1981) experimental evidence has indicated that inflammation increases the levels of dolichol-linked intermediates and the phosphorylation of dolichol by CTP-dependent dolichol kinase.

Figure 58 shows the effects of starvation, refeeding, and inflammation upon the hepatic levels of total lipid and cholesterol. Starvation had little effect on either total lipid or cholesterol, decreasing the former and increasing the latter slightly. Refeeding a fat-free diet caused a gradual increase in both cholesterol and total lipid levels. Inflammation at 0 h of refeeding caused a much more rapid increase in both cholesterol and total lipid in the first 12 h after injection but this initial increase declined slightly to approximately the level of the non-inflamed animals. This agrees with the observation by Coolbear and Mookerjea (1981) of a peak in dolichol-kinase activity at 12 h after inflammation.

Figure 59 shows the effects of starvation, refeeding and inflammation upon the levels of hepatic triglyceride (total lipid levels from Fig. 58 are included for comparison). As expected the levels of triglyceride declined rapidly with starvation while refeeding caused a rapid increase

Figure 58: Effects of starvation, refeeding and inflammation upon hepatic total lipid and cholesterol levels.

Rats were maintained on a normal diet, starved for 48 H and refed a fat-free diet for 12 ar 24 H. Inflammation was induced at 0 H of refeeding (after 48 H starvation) and is represented by the solid symbols. Values for non-inflamed animals are given by the open symbols. Cholesterol levels (△—△, ▲--▲) and total lipid levels (○—○, ●--●) were determined as described in Experimental Procedures. Each point represents an average value obtained using 3 rats.

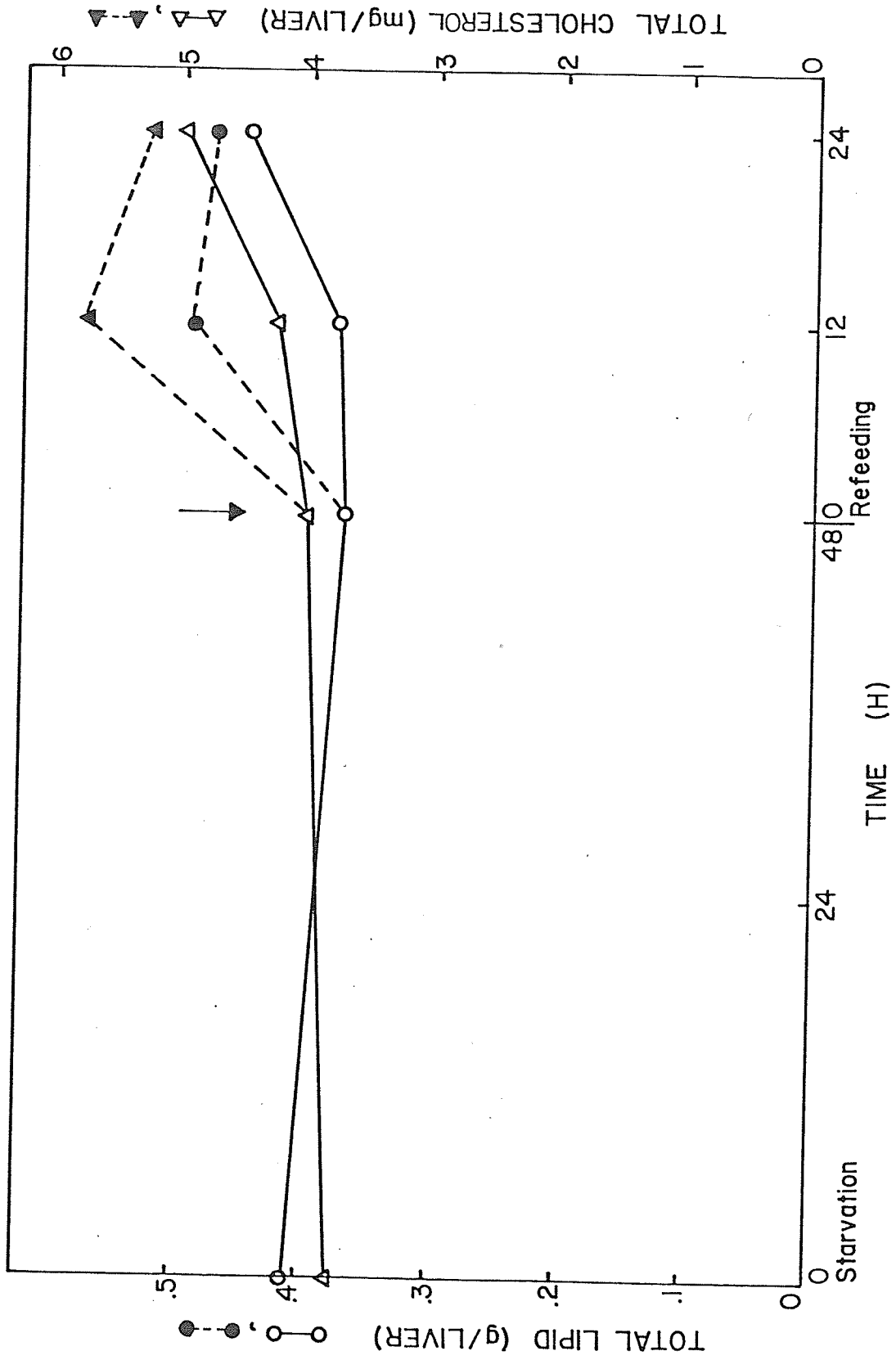

TOTAL CHOLESTEROL (mg/LIVER)
TOTAL LIPID (g/LIVER)
TIME (H)
Starvation
Refeeding
0
24
48
0
12
24
0
1
2
3
4
5
6
.1
.2
.3
.4
.5

Figure 59: Effects of starvation, refeeding and inflammation upon hepatic total lipid and triglyceride levels.

Further details are given in the legend to Figure 58. Triglyceride levels (□—□, ■--■) and total lipid levels (○—○, ●--●) were determined as described in Experimental Procedures.

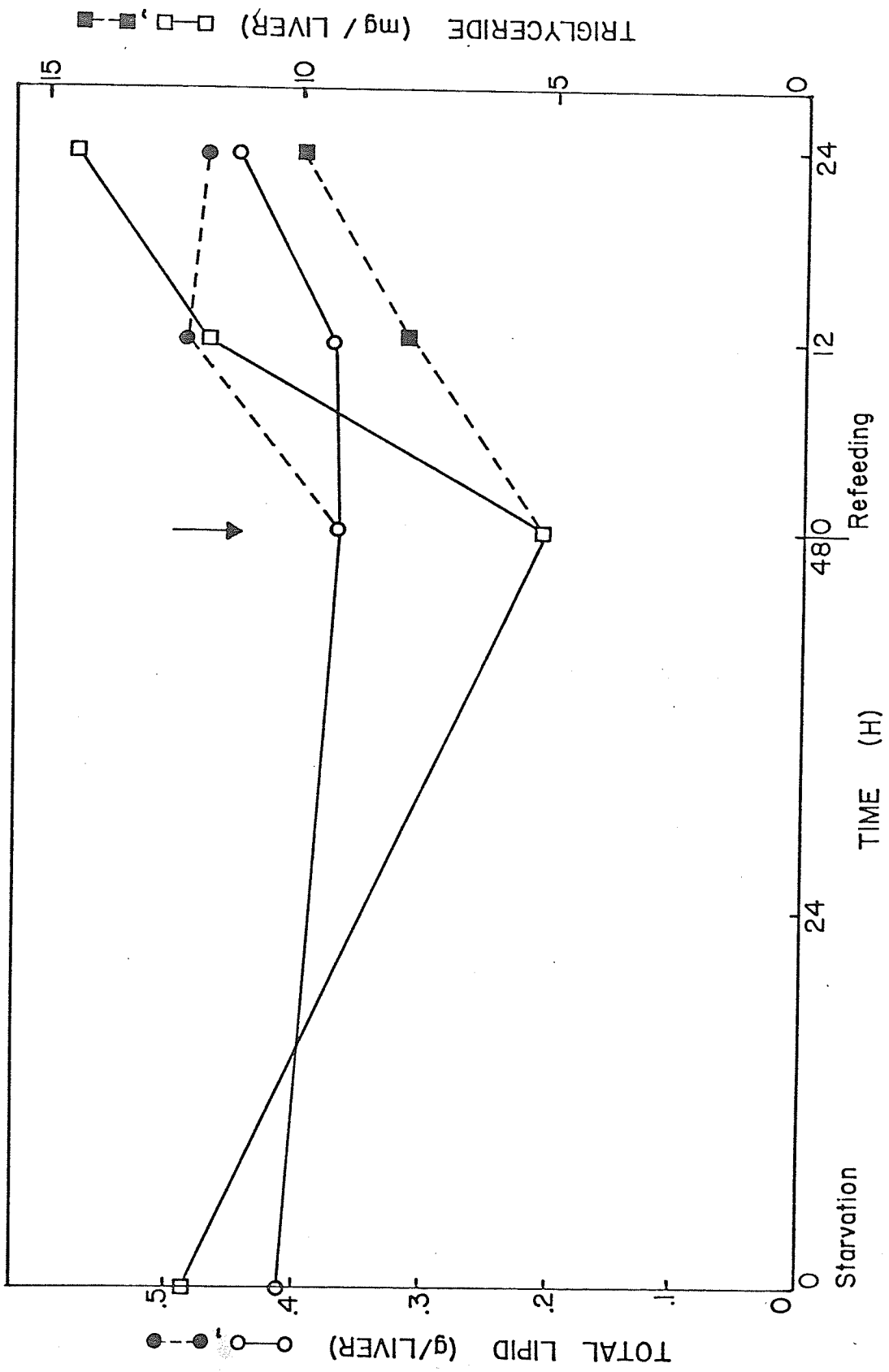

TRIGLYCERIDE (mg / LIVER)
TOTAL LIPID (g/LIVER)
TIME (H)
Starvation
Refeeding
0
24
48
0
12
24
0
5
10
15
1
2
3
4
5

Table 16.

Effect of Adaptive Synthesis and Inflammation on the Hepatic Levels of Various Lipids.

Duration of fat-free feeding (H)[a]	Total Lipid		Cholesterol		Triglyceride	
	g liver	mg g liver	mg liver	mg g liver	mg liver	mg g liver
0	0.370	41.8	4.0	0.45	5.65	0.64
12	0.374	26.5	4.26	0.30	12.0	0.85
12*	0.486	34.7	5.6	0.40	8.5	0.60
24	0.464	25.5	5.0	0.28	14.1	0.75
24*	0.476	33.3	5.25	0.38	10.5	0.70
Normal Diet	0.41	30.3	3.75	0.28	12.03	0.90

[a]Rats were starved for 48 H prior to refeeding a fat-free diet.

*Indicates animals were inflamed at 0 H of refeeding.

to above normal levels by 24 h of refeeding. Inflammation suppressed this increase by approximately 50%. Table 16 summarizes these results and expresses them as both per liver and per gram wet weight of liver.

6.1 Effects of Adaptive Synthesis and Inflammation upon the levels of Acetyl-CoA Carboxylase.

Figure 60 shows the effect of adaptive synthesis and inflammation upon the levels of hepatic acetyl-CoA carboxylase. Refeeding for 24 h after a 48 h starvation caused an approximately 15-fold increase in enzyme activity. This increase is similar to that observed for FAS. Inflammation at 0 h of refeeding resulted in a suppression of enzyme activity but not nearly to the degree observed for FAS. This may indicate a differential effect of inflammation on these enzymes which are both involved in the lipogenic route producing fatty acids.

6.2 Effects of Adaptive Synthesis and Inflammation on the levels of ATP-Citrate Lyase.

Figure 61 shows the effect of adaptive synthesis and inflammation upon the levels of hepatic ATP-citrate lyase. Refeeding a fat-free diet caused a 25-fold increase in enzyme activity which was suppressed to a large extent by inflammation at 0 h of refeeding. This pattern of suppression more closely resembles that observed with FAS (Fig. 11) than was found for acetyl-CoA carboxylase.

These differential effects of inflammation will be discussed in more depth in the Discussion section of this thesis.

Figure 60: Interaction between experimental inflammation and adaptive synthesis of acetyl-CoA carboxylase.

Rats were starved for 48 H and refed a fat-free diet for 12 and 24 H. Inflammation was induced at 0 H of refeeding and is represented by the solid symbols. Values from non-inflamed animals are given as the open symbols and the level in rats maintained on a normal diet is given by n.d.———. The assay is described in Experimental Procedures. Each point represents an average value obtained using 3 rats with the enzyme activity expressed as nanomoles of malonyl-CoA produced per minute per liver. Individual values varied from this average by less than $\pm$ 10%.

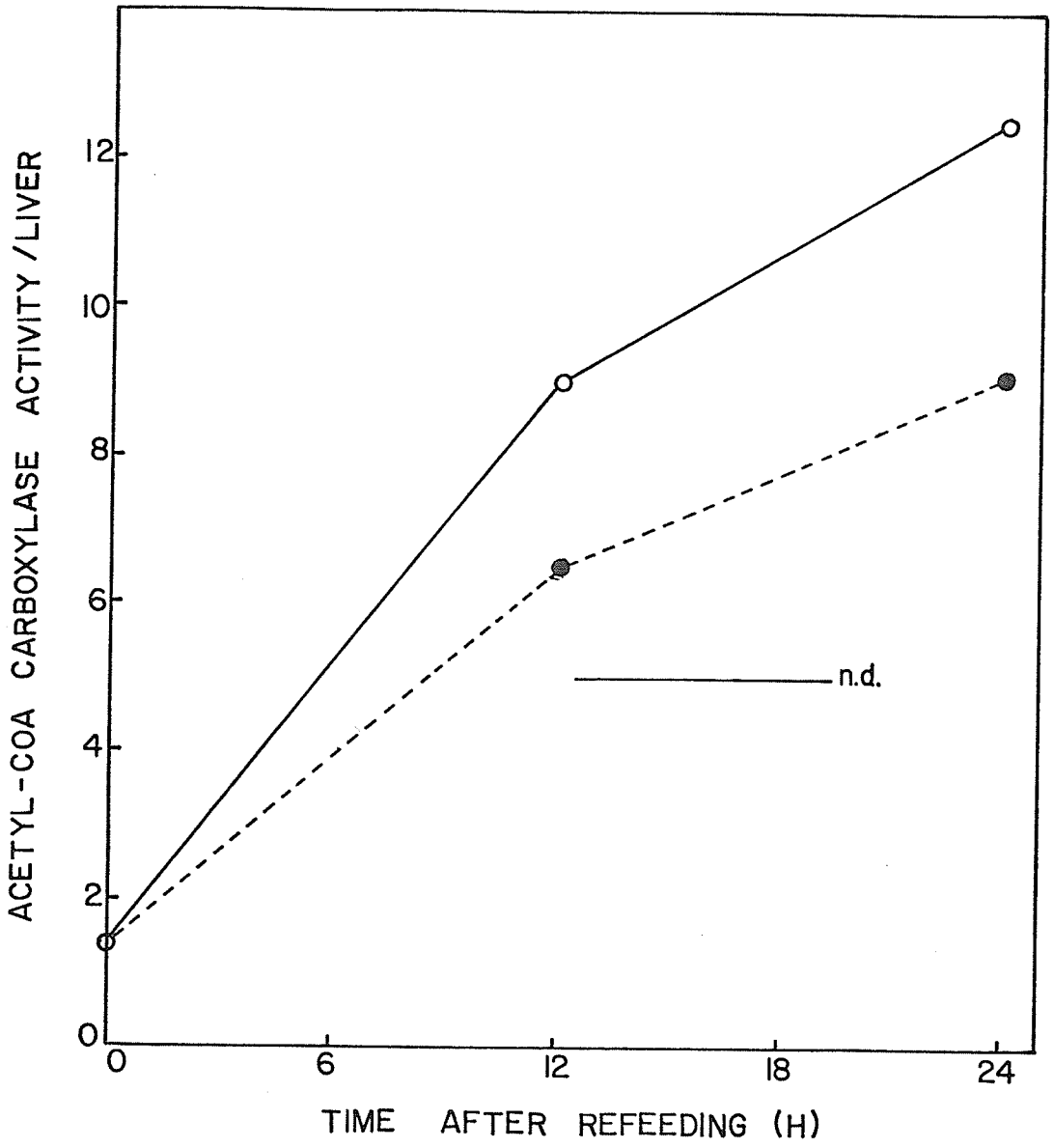
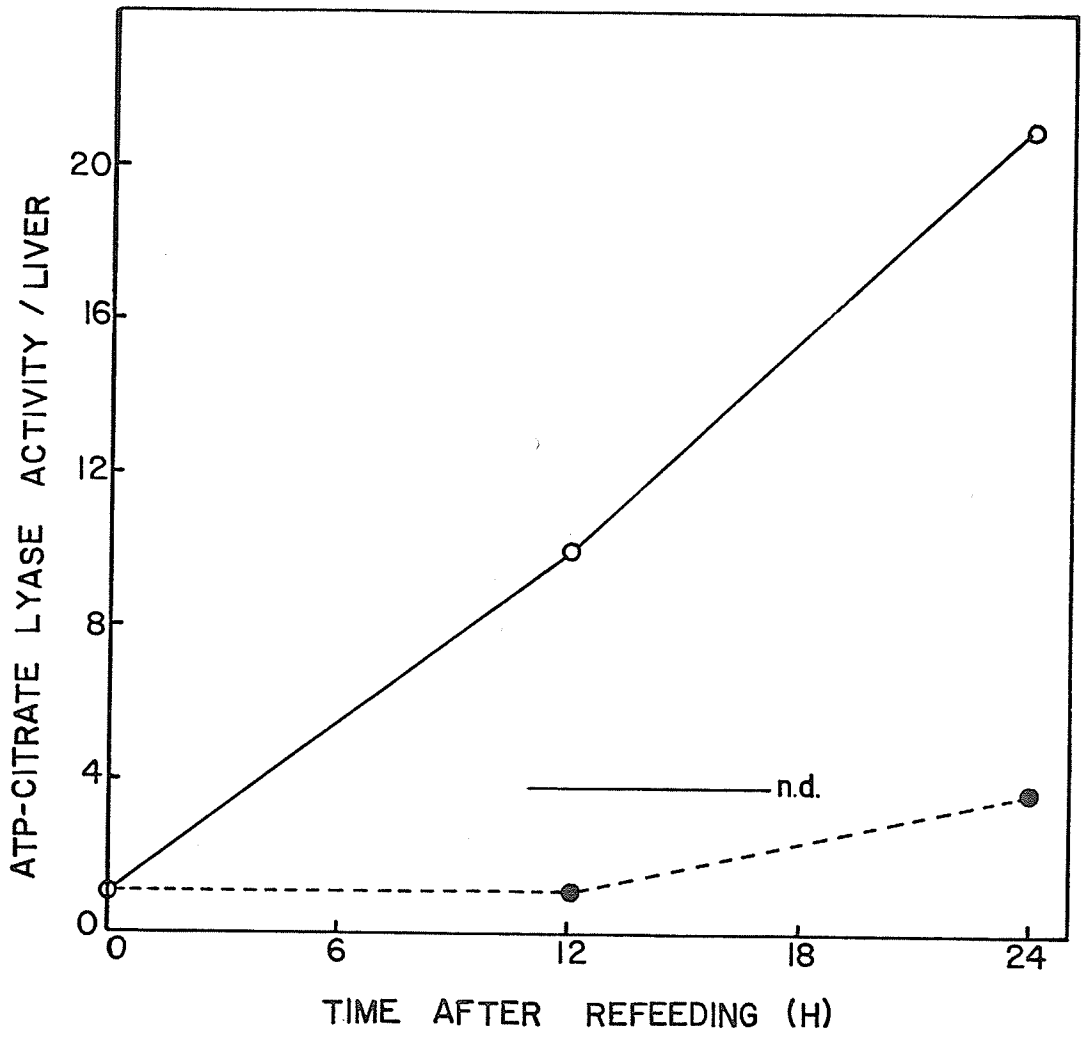

ACETYL-COA CARBOXYLASE ACTIVITY /LIVER
0
2
4
6
8
10
12
0
6
12
18
24
TIME AFTER REFEEDING (H)
n.d.

Figure 61: Interaction between experimental inflammation and adaptive synthesis of ATP-citrate lyase.

Further details are given in the legend to Figure 60.

Enzyme activity is expressed as moles of NADH oxidized per minute per liver.


ATP-CITRATE LYASE ACTIVITY / LIVER
20
16
12
8
4
0
n.d.
0
6
12
18
24
TIME AFTER REFEEDING (H)

DISCUSSION

DISCUSSION

1. THE MECHANISM OF THE EFFECTS OF EXPERIMENTAL INFLAMMATION ON ADAPTIVE SYNTHESIS OF RAT LIVER FATTY ACID SYNTHETASE.

1.1 Variations in the Levels of FAS Protein

Early evidence indicated the rapid rise in hepatic fatty acid synthetase levels observed with refeeding was due to an increase in the levels of enzyme protein. Hicks *et al.* (1965) prevented the adaptive synthesis of hepatic FAS by administration of puromycin and actinomycin D at the beginning of the refeeding period. Burton *et al.* (1969) confirmed this result by showing the actual amount of enzyme isolated from the livers of fasted, normal and refed rats varied according to the nutritional status of the animal. A previous report from this laboratory (Shutler *et al.*, 1977) showed that the increase in liver FAS activity characteristic of adaptive synthesis was suppressed by induction of inflammation (see Fig. 11). The results presented here confirm the earlier observations on increases of enzyme protein during fat-free diet refeeding and indicate that suppression of adaptive synthesis by inflammation was accompanied by a suppression of the levels of enzyme protein.

Figures 12, 13 and 14, and Table 6 show that the immunochemical equivalence points of FAS preparations derived from both inflamed and non-inflamed rats killed at various times after refeeding, and with different intervals between refeeding and inflammation and inflammation and sacrifice, are essentially identical, despite the wide variations in the specific enzyme activity of the preparations. This indicates that all FAS molecules have similar catalytic abilities, and that suppression of FAS activity by inflammation resulted from decreased

content of the enzyme.

This conclusion is confirmed by the data in Results (Section 2.2), which shows the hepatic concentration of material cross-reacting with anti-FAS serum (presumably FAS) varies in concert with FAS activity. The amount of FAS estimated by radial immunodiffusion was greater than the amount calculated from the enzyme activity, assuming a specific activity of 1 μmol of NADPH oxidized per minute per mg. Despite this discrepancy the experiments indicate that the changes in FAS activity reflected changes in the amount of enzyme present.

Table 7 summarizes the results from the experiments determining the effects of inflammation upon FAS activity and amounts of cross-reacting material. The suppression of FAS activity by inflammation was closely paralleled by the suppression of cross-reacting material. The degree to which FAS activity was suppressed by inflammation is apparently related to both the duration of fat-free feeding prior to the induction of inflammation and the interval between inflammation and sacrifice. These trends are illustrated in Figure 31 for rats killed 15, 24 and 48 h after the start of refeeding. For rats killed 15 and 24 h after the start of adaptive synthesis, the suppression of fatty acid synthetase by inflammation decreased as the interval between the start of refeeding and the injection of oil of turpentine increased (Fig. 31A). These results agree with the observations of Shutler *et al.* (1977). Comparison of the suppression of synthetase activity in animals killed 15 and 24 h after refeeding, having different intervals between inflammation and sacrifice (Fig. 31B), indicates suppression was greater in animals having longer exposure to the inflammatory stimulus.

The longer period of refeeding (48 h) shows results that differ slightly from the 15 and 24 h refeeding periods. The longer the time between refeeding and inflammation the greater the degree of suppression although the difference between the 0 and 10 h injection times is only 15% compared to a 40% decrease in the degree of suppression for the 15 h refeeding experiment. Similarly the longer the time between inflammation and sacrifice for the 48 h refed rats, the less the degree of suppression by inflammation.

These results imply the existence of complex cellular controls that recognize the inputs of two different external stimuli and try to adjust the cellular metabolism to compensate for both. After 48 h of starvation the cellular metabolism has reached a steady-state (see following discussion on nutritional deprivation and inflammation) which is an attempt to compensate for continued nutritional deprivation. Induction of adaptive synthesis by refeeding causes rapid changes in the metabolic mechanisms of the body, which, if interfered with by inflammation in the early stages of refeeding, are suppressed to a large degree (see Fig. 31A). If inflammation is a later event (i.e. 5 or 10 h after the start of refeeding) it requires a much longer time interval for the interference to become apparent. An early injection time followed by 48 h of refeeding shows a decrease in inflammatory suppression indicating that the high degree of interference in the early stages of refeeding may have compensated for the inflammatory stimulus and the animal may be beginning a late-adaption to fat-free feeding.

Jamieson and Ashton (1973a) showed that hepatic α_1-acid glycoprotein, an acute-phase reactant, increased about 5-fold at 12 h following the induction of inflammation in rats maintained on a normal

diet. Shutler *et al.* (1977) showed that adaptive synthesis interfered with the inflammatory response. If inflammation was an early event in adaptive synthesis, α_1-acid glycoprotein and albumin levels increased in a manner characteristic of inflammation alone. If inflammation was a late event after refeeding this increase was suppressed. This data indicates there is a complex competition occurring between the cellular processes initiated by adaptive synthesis and inflammation.

A recent study has found increased levels of fatty acid synthetase and acetyl-CoA carboxylase with bacterial infections in fasting rats (Pace *et al.*, 1981). The response of animals to fasting is a complex process involving a hierarchy of priorities which are determined by the immediate physiological needs of the body (for reviews see Cahill *et al.*, 1967; Krebs, 1972). The glycogen supply is exhausted in less than 24 h of fasting and there is a mobilization of free fatty acids through ketogenesis and of free amino acids derived from the proteins of skeletal muscle and other peripheral body tissues. Neufeld *et al.* (1980) found that inflammation and infection in fasted rats caused an inhibition of ketogenesis and a depression of the levels of plasma free fatty-acids. They have demonstrated that this decreased ketone body synthesis may be related to alterations in the mitochondrial and cytosolic pools of carnitine and coenzyme A, which suggests some form of control over compartmentalization (Pace *et al.*, 1980). Increased levels of cytosolic citrate and decreased mitochondrial citrate were also observed leading to the suggestion that infection-related decreases in ketogenesis are a result of increased triglyceride formation (i.e. decreased oxidation of long chain fatty acids) and an increased shuttling of acetyl-groups to the cytosol for

synthetic processes (Pace *et al.*, 1981). This led to the authors suggesting a fatty-acid futile cycle in the liver of infected-fasting rats. These results suggest further complexities in the interrelationships occurring with starvation, refeeding and inflammation. A possible explanation of the increase in lipogenesis during fasting and inflammation may involve an increase in membrane production which is known to occur during inflammation alone (see Lewis, 1980; Lombart *et al.*, 1980). An interesting possibility exists that the body increases its mobilization of proteins to supply amino acids for glucose production to compensate for the stress of starvation while increasing the lipogenic pathways leading to membrane formation to increase hepatic capabilities to secrete acute-phase reactants to compensate for the stress of infection.

Evidence indicates that the variations observed in FAS and α_1-acid glycoprotein levels are due to variations in the amounts of each protein present. This implicates the processes which control cellular protein levels as the mechanisms controlling the variations observed.

1.2 Effects of Adaptive Synthesis and Inflammation on the Turnover of Hepatic FAS.

1.2.1 Variations in the relative rates of FAS synthesis.

Both adaptive synthesis and the response to inflammation are known to involve increases in the rates of synthesis of specific proteins (Burton *et al.*, 1969; Craig *et al.*, 1972; Jamieson and Ashton, 1973). Table 5 (from Volpe and Vagelos, 1976) shows the variations in FAS activity and the corresponding changes in the rates of synthesis and degradation, during different nutritonal, hormonal, developmental and genetic conditions. Variations in the rates of synthesis and degradation have been observed with fasting for FAS and

a number of other lipogenic enzymes (see Historical).

The results presented in Figure 33 indicate that starvation for 48 h resulted in a 6-fold decrease in FAS activity which was accompanied by a decrease in the rate of synthesis of 33%. This implicates the occurrence of degradative increases which agrees with previous results (Volpe *et al.*, 1973). Refeeding a fat-free diet for 48 h resulted in a maximal 25-fold increase in enzyme activity and a maximal 10-fold increase in the relative rates of enzyme synthesis, over the starvation levels. The increase over the levels observed in rats fed a normal diet was 8-fold for activity and 6-fold for relative synthesis indicating that refeeding may bring the rates of enzyme degradation back to normal levels, and the increase over normal levels may be accounted for by an increase in the relative rates of enzyme synthesis. These results are slightly different from earlier studies which largely accounted for the increased levels of enzyme over starvation levels by an increased level of enzyme synthesis (see Volpe and Vagelos, 1976; Nepokroeff and Porter, 1978). The levels of relative synthesis attained with refeeding are similar to levels attained in other experiments. Joshi and Sidbury (1976) found a 25-fold increase in hepatic FAS activity in glucagon-treated chick embryos and correlated this to an increase in the relative rate of synthesis (% of total protein) from 0.2 to 7.6. Flick *et al.* (1977) found a two-fold increase in activity in rats starved for 48 h and refed a fat-free as compared to a fat-supplemented diet. The increase in activity occurred with a change in relative synthesis of 6% to 14%.

Longer periods of refeeding (up to 162 h) caused a decrease in activity to normal levels accompanied by a corresponding decrease in synthesis to normal levels (Fig. 34 and 35). Induction of inflammation

at the start of refeeding caused a suppression of the adaptive synthesis of FAS activity and a corresponding depression in the observed increase in the relative rate of enzyme synthesis. After 24 h of refeeding the inflamed animals had 1/3 the hepatic enzyme activity of the non-inflamed animals. This correlated with a 1/3 depression in the relative rate of synthesis at 24 h (Fig. 33). Inflammation induced at a time when enzyme activity was returning to normal levels (62 h after the start of refeeding) caused an accelerated decrease in the activity. This resulted in enzyme levels which were 1/3 the level observed in refed rats (Fig. 34). Inflammation at 62 h also resulted in an accelerated depression of the relative rates of synthesis (Fig. 35), although the decrease cannot completely account for the decreased level of activity. This again implies the existence of enzyme degradative increases which enhanced the decrease in enzyme levels.

The patterns observed in Figure 33 for the activity and rate of synthesis of inflamed rats are also interesting. Inflammation at 0 h of refeeding was accompanied by a rapid increase in the relative rate of FAS synthesis which reached a maximum after 12 h of refeeding and maintained that level until at least 48 h of refeeding. The activity increase observed with inflammation followed a different pattern. There was an initial increase in activity followed by a long lag phase with a gradual increase in activity which reached a maximum after 48 h of refeeding. This implies the existence of degradative increases occurring while the activity and synthesis were gradually increasing. This is interesting from the standpoint that the only previous observation of a simultaneous increase in synthesis and degradation was with Vitamin B_{12} deficiency (Frenkel *et al.*, 1974).

Other investigations found that increases in enzyme activity were accounted for by increased FAS synthesis and that only decreases in activity (i.e. fasting and diabetes) were accompanied by increased rates of degradation (see Volpe and Vagelos, 1975). This led to the prediction that the mechanisms of degradation were controlled by the mechanisms of synthesis which is unlikely considering the nature of the processes involved. Another interesting observation was the rapid initial rise in both activity and synthesis observed in inflammed rats after inflammation at 0 h of refeeding. This may be related to the increases observed by Pace *et al.* (1981) during infection of starved animals. This may indicate the existence of two separate controls on the mobilization of the protein-synthesizing mechanisms of the cell.

The mechanism and control of the hepatic cellular protein-synthesizing apparatus was briefly outlined in the Historical. Its complexity is increased by the existence of two functionally-different and physically separated populations of ribosomes which may have similar or different mechanisms of control.

Shutler *et al.* (1977) found shifts in the protein-synthesizing machinery with adaptive synthesis and inflammation. FAS and α_1-acid glycoprotein are known to be synthesized primarily on "free"-ribosomes (Yu and Burton, 1975) and "membrane-bound" ribosomes (Jamieson, 1977), respectively. Shutler *et al.* (1977) found that the ratio of free/bound ribosomes increased with the duration of refeeding. Inflammation induced 2.5 h after the start of refeeding suppressed this increase in the free/bound ratio suggesting the occurrence of a control operating over the ribosomal-membrane association-disassociation. The synthesis of FAS on membrane-free polysomes has been confirmed by Nepokroeff

et al. (1979) for rat liver FAS and Zehner *et al.* (1977) for the avian liver enzyme. These results agree with the proposal that secreted proteins are synthesized on polysomes associated with the membrane while cytoplasmic proteins are synthesized on free-polysomes (see Historical). These results may also be partially explained in terms of the "signal hypothesis" (Blobel and Dobberstein, 1975) in which the signal for membrane-association resides in the messenger RNA. If inflammation causes a shift in ribosome distribution away from free polysomes, the initial increase in FAS synthesis (for inflamed animals in Fig. 33B) may be occurring prior to the transcriptional activation for production of the messenger RNA for acute-phase proteins which would control the membrane-association increase. This implies the existence of either some form of translational activation with adaptive synthesis or a differential transcription or post-transcriptional control of mRNA production for acute-phase and lipogenic proteins. Zahringer *et al.* (1976) found that iron administration to rats resulted in a shift of ferritin mRNA from the post-ribosomal cytosol into free-polyribosomes for translation indicating other factors besides the mRNA are involved in polyribosome association (see Historical).

Ribosome content can also be modulated, and for long-term adaptions in the rate of hepatic protein synthesis (i.e. during long-term starvation) this has been suggested as a major regulatory mechanism. Fasting animals exhibit a rapid decline in hepatic ribosome levels over the initial 24-48 hours of starvation which has been suggested as a long-term adaptive process which is not involved in rapid changes in the rates of protein synthesis (for review see Henshaw, 1980). Starvation is also known to modulate the activity of

ribosomes through an inhibition of the initiation and elongation processes of translation (Millward *et al.*, 1973). Refeeding a mixture of essential amino acids has been observed to increase the aggregation of rat liver ribosomes into polysomes (Clemens and Pain, 1974). Conde and Franze-Fernandez (1980) found that refeeding mice restored the levels of ribosomes through enhancement of synthesis and suppression of degradation of ribosomal components. The authors also found the transcriptive capacity of liver nuclei is unimpaired after protein deprivation. In fact with more severe starvation RNA polymerase I activity was found to increase by 30%, and a further increase (2-fold) with refeeding was observed. Others have found a decrease in liver nuclei polymerase activity with starvation in rats (Coupar *et al.*, 1978).

These results suggest the existence of transcriptional controls which will be discussed at length in the section on mRNA variations.

Conde and Scornik (1976) found that total protein synthesis in mouse liver was lowered by feeding a protein-depleted diet, but it recovered to above-normal levels after refeeding. Total protein breakdown was found to be greatly depressed during the refeeding period. The current view now emerging is that control of protein levels through changes in the rates of degradation have been underemphasized due to the experimental difficulties encountered.

1.2.2 Variations in the relative rates of FAS degradation.

Original studies found that the increase in FAS levels with refeeding could be accounted for by an increase in the rate of synthesis and no change in the relative degradation of the enzyme. A summary of these results is presented in Table 17. Starvation

Table 17.

Comparison of the Half-Life of FAS and Total Protein under Different Conditions.

Experimental Description	Half-Life FAS (h)	Half-Life Total Protein (h)	References
Normal Diet	69		Craig *et al., 1972*
Starved 48 H	42		
Refed fat-free diet	69		
Fed fat-free diet	91	77	Flick *et al., 1976*
Fed fat-supplemented diet	46	100	
Normal diet	70	108	Volpe *et al.*, 1973
Starved	18	89	
Refed fat-free diet	65	104	
Normal	63		Frenkel *et al.*,1974
B_{12} Deprived ($k_s \uparrow$)	35		
B_{12} Supplemented	65		

resulted in a decrease in the half-life of total protein and FAS (Volpe *et al.*, 1973) but refeeding caused a decrease in degradation only to the extent found in animals fed a normal diet. This led to the prediction that the "overshoot" observed in the levels of lipogenic enzymes with refeeding is dependent solely upon a mobilization of the cellular protein-synthesizing mechanisms. More recent studies with differentiating preadipocytes have resulted in similar conclusions. Student *et al.* (1980) observed a 20-fold increase in FAS activity with differentiation of preadipocytes into mature adipocytes containing large amounts of deposited triglyceride. The rate of incorporation of a non-decaying label into immunoprecipitable FAS was found to increase by a similar amount while the half-life remained constant at 1.4 days. Weiss *et al.* obtained similar results from the same system. Procsal *et al.* (1976) have reached the same conclusions for dietary induction of rat liver 6-phosphogluconate dehydrogenase.

The results presented in Table 8 and Figure 36 agree with previous suggestions that degradative influences are important during periods when the rate of enzyme synthesis is decreasing. The half-life of rats inflamed at 62 h of refeeding a fat-free diet and refed for a further 100 h was approximately 50% that of the non-inflamed animals refed for the same period. The total protein half-life decreased by 15% indicating inflammation may have induced a more rapid turnover of lipogenic enzymes, including FAS, which is not compensated for by the increase in acute-phase reactants. This is probably an underestimation as an increase in secretable protein synthesis and secretion from the liver should result in a depletion of the liver protein pool. This leads to a prediction of increasing synthesis of acute-phase reactants while degradation of lipogenic enzymes is increasing.

Comparison of relative degradation of FAS through the turnover index given in Table 10 results in similar conclusions. For the time periods chosen, the relative rates of synthesis were approaching a steady-state for both inflamed and non-inflamed animals indicating the variations must be due to changes in the decaying-label levels for FAS or total protein. The data indicates a more rapid turnover of FAS occurs in the early periods for inflamed rats with the turnover-index for both inflamed and non-inflamed animals becoming approximately the same by 162 h of refeeding (Fig. 37). Thus the changes in degradative effects appear to account for the 50% decline in FAS activity levels (Fig. 34) with only a 33% decline in the relative synthesis of FAS (Fig. 35).

The results in Tables 9 and 10 and in Figure 37 for the first 24 h of refeeding also suggest the interaction of degradative influences in both inflamed and non-inflamed animals. The suggestion of degradative increases, discussed in the previous section, for inflamed animals appears to agree with the observed high turnover and high-turnover index found after 12 h of refeeding (Fig. 37). The turnover index for non-inflamed animals remained constant but interpretation of these values during non-steady state conditions indicated the relative rate of FAS degradation decreased in the first 24 h of refeeding. Rearranging the turnover index equation (shown in section 3.2.2 of the Results) indicates that a 16-fold increase in relative synthesis would require a 16-fold decrease in the value representing FAS degradation/Total Protein degradation to maintain the turnover index at a constant level. Table 9 indicates that as the relative rate of synthesis of FAS compared to total protein increased by 16-fold the turnover increased almost 2-fold

indicating the level of decaying label increased at least 8-fold. These results imply an increase in degradation during the early stages of refeeding for inflamed animals and a decrease in degradation for non-inflamed animals. Recently evidence has accumulated indicating degradative changes may be the most important parameter determining an increase in protein content (Conde and Scornik, 1977).

Starvation and refeeding experiments have given variable results using different methodologies for studying degradation and synthesis (for review see Garlick, 1980). Indications from these experiments seem to implicate degradative influences as extremely important. Agustine and Swick (1977) observed no increase in protein synthesis soon after refeeding when protein mass was increasing rapidly, and from measurements of the loss of label from protein at short times after $NaH^{14}CO_3$ injection predicted a large decrease in degradative rates. Interpretation of results is complicated by observed differences in the effects of starvation and different levels of dietary-protein on the rates of synthesis and degradation of proteins.

Paskin and Mayer (1978) found that hormonal stimulation of FAS accumulation in explants of rabbit mammary glands was due to a decrease in the rate of enzyme degradation not an increase in the rate of synthesis. Upon hormone removal the degradation rate increased and the levels of FAS decreased. The same authors predicted degradative changes because of the advantages to the cell in terms of energy requirements and the rapidity of response (Paskin and Mayer, 1977). They also predicted that a differential response of different classes of proteins occurred during cytodifferentiation. This differential response is based upon a predicted physical separation of membrane or secretable

proteins and the cytoplasmic proteins. A differential response depending upon the cellular requirements for different groups of internal proteins has also been predicted.

Dice *et al.* (1978) proposed a two-component degradative mechanism occurring in diabetes or nutrient deprivation. The two proteolytic systems included non-lysosomal and lysosomal components with degradative controls existing on either or both. Mortimore *et al.* (1975) have related alterations in the lysosomal system to changes in degradation. When protein breakdown was enhanced (i.e. starvation), the lysosomes became more sensitive to osmotic shock (membrane-changes) and this effect could be suppressed by insulin and amino acids. The authors also observed an increase in the numbers of enlarged lysosomes, with electron microscopy. Non-lysosomal proteases have also been found to increase during starvation and diabetes (see Goldberg and St. John, 1976). Dice *et al.* (1978) suggest some control may exist over the protease sensitivity of groups of proteins. The two-component degradative system has been discussed by Wilde *et al.* (1980). They compared degradation of fatty acid synthetase and casein, a secretable protein, in mammary gland explants. Using ammonium chloride, which is considered to specifically inhibit autophagic protein degradation by lysosomes, the increase in FAS degradation with hormone removal was partially inhibited. Other groups have observed ammonium chloride inhibition of degradative increases in rat hepatocytes (Seglen *et al.*, 1979) and cultured fibroblasts (Amenta *et al.*, 1978). Inhibition of casein degradation (Wilde *et al.*, 1980) by ammonium chloride was not observed until long after the hormone removal. This may be due to the differential locations of the two proteins and may indicate the existence of different mechanisms of control over degradation for different

groups of proteins.

These differential degradative systems may be an explanation for the rapid increase observed in both synthesis and degradation (Fig. 33 and 37) for inflamed animals while the level of FAS activity remained low.

1.3 Effects of Adaptive Synthesis and Inflammation on the Levels of Translatable Messenger RNA.

From the previous section it appears evident that a complex set of separate and interrelated mechanisms exist which control the levels of FAS during starvation, adaptive synthesis and inflammation. The 25-fold increase in FAS activity observed after 48 h of refeeding and the suppression by inflammation (Fig. 33) can be at least partially explained by variations in the relative rates of enzyme synthesis. The control over changes in protein synthesis (discussed previously in the Discussion and Historical) can reside in either transcription, translation or the processes in between. Earlier experiments showed variations in ribosome levels and distribution, (see previous Discussion), indicating at least some form of translational control, although these may be important only as a stabilization of long-term adaptory changes.

Nepokroeff and Porter (1978) and Flick *et al.* (1978) found that the increase in hepatic FAS activity with refeeding correlated with increases observed in the levels of translatable messenger RNA. Flick *et al.* (1978) translated RNA, isolated by immunoprecipitation of polysomes with anti-FAS antiserum, in a wheat germ cell-free protein-synthesizing system. They isolated a major polypeptide, with an approximate molecular weight of 225,000, which contained 10% of the label precipitated by trichloroacetic acid (after 12 h of refeeding). This is similar to

the results obtained in our study, the polypeptide isolated by electrophoresis had a molecular weight of 220,000 to 240,000 (Fig. 46 and 48) and at the time of maximum induction contained 7.5% of the label found in total protein. Nepokroeff and Porter (1978) translated poly(A)-mRNA in *Xenopus laevis* oocytes and isolated FAS by immunoprecipitation. They found a maximal increase in FAS mRNA activity after 12 h of refeeding, a time of refeeding which also exhibited the maximal increase in the rate of enzyme synthesis. Table 15 illustrates that the maximal increase observed with refeeding, in our system, occurred after 24 h. Suppression of the rate of FAS synthesis by inflammation during adaptive synthesis caused a decrease in the FAS mRNA/Total Protein mRNA ratio of 60%. This correlates extremely well with the 65% decrease observed in the relative rate of enzyme synthesis (Fig. 33).

Observation of the results indicates that the 25-fold increase in activity for non-inflamed animals after 48 h of refeeding is accompanied by a maximum 12-fold increase in the rate of synthesis and possibly a 2-fold decrease in the relative degradation. To account for a 12-fold increase in the relative protein synthesis, the proportion of FAS mRNA increases only 5-fold which implies some form of translational control. A similar comparison can be made for rats inflamed at 0 h of refeeding. The level of activity increased by 12-fold and the rate of synthesis by approximately 4-fold (and increased degradation compared to non-inflamed animals). The proportion of mRNA specific for FAS increased only 2-fold which again implicates the occurrence of translational controls. Since the relationships between the levels of mRNA, enzyme synthesis and activity were very similar

for both non-inflamed and inflamed animals the possibility exists that the translational factor is the same in both cases with only the degree of stimulation altered by inflammation. The existence of translational controls has recently been proposed to account for the increased synthesis of a secretory protein from rat seminal vesicles (Kistler *et al.* 1981).

Interesting results for total protein synthesis from poly(A)-mRNA are illustrated in Figure 50. Low levels of activity were observed for messenger RNA from starved animals compared to animals fed a normal diet or refed a fat-free diet after a period of starvation (both non-inflamed and inflamed animals). These results do not agree with those of Cladavas and Cottam (1981) who found the levels of TCA precipitable counts to be identical for equal amounts of poly(A)-mRNA from starved, refed or non-treated rats. Lee and Englehart (1978) found growth-related fluctuations in the make-up of messenger RNA and predicted that a reduction in the utilization of existing mRNA should occur during growth cessation. Mori *et al.* (1981) also observed a decrease in total translational activity in fasted rats as compared to normal animals. They also observed an increase in the mitochondrial urea cycle enzymes with starvation and found a decrease in specific mRNA levels with a large decrease in specific enzyme degradation accounting for the enzyme increases.

The low levels of activity in mRNA from starved animals may be due to a differential purification and carry-over of some translational inhibitor or possibly by a differential translation of some type of poly(A)-containing mRNA. Lau *et al.* (1979) found that a variety of conditions, (K^+, Mg^{+2}, tRNA levels), specifically affected the translation of FAS mRNA while not affecting the total protein translation.

This implies some form of differential translational control which resides in the added poly(A)-mRNA. These differences with varying conditions were not observed in the work described here.

Figures 39 and 40 indicate the existence of differing populations of poly(A)-mRNA from inflamed and non-inflamed animals. Further investigations are required to determine if starvation results in a different population of messenger RNA which might have reduced translational characteristics. The causes of differential translation of poly(A)-mRNA might involve bound small-RNA's (Bag *et al.*, 1980) which copurify with the message, physical variations in the poly(A) or methyl groups associated with the message, partial degradation of some portion of the message, etc. Wallace *et al.* (1979) found differential translational activity of messenger RNA from unstimulated and phytohaemagglutinin-stimulated lymphocytes.

The differences observed in Figures 39 and 40 between populations of poly(A)-mRNA may indicate some form of size control exerted at the level of transcription. Inflammation caused a decrease in the larger poly(A)-mRNA species and an increase in the smaller regions. Figure 49 illustrates the electrophoretic pattern of the total translation products with poly(A)-mRNA from both inflamed and non-inflamed animals. There was an increase in the smaller molecular weight proteins produced in the inflamed case and an increase in the larger molecules produced in non-inflamed animals. These results agree with the observed differences in poly(A)-mRNA and predictions of protein sizes produced. Table 18 summarizes the subunit molecular weights of some lipogenic enzymes and some acute-phase reactants. Observation indicates the sizes of the lipogenic enzymes are much larger than the sizes of the acute-phase reactant proteins and would require mRNA

Table 18.

Molecular Weights of Various Lipogenic Enzymes and Acute-Phase Reactants.

Lipogenic* Enzymes	Subunit Molecular Weight	(#)	Molecular Weight of Native Protein
Fatty acid synthetase	250,000	(2)	500,000
Acetyl-CoA carboxylase	118-125,000	(2)	215,000
Glycogen synthetase	90,000	(3)	250,000
ATP-citrate lyase	290,000	(2)	575,000
Phosphofructokinase	400,000	(2)	800,000
	210,000	(2)	400,000
	100,000	(2)	210,000
	60,000	(2)	100,000
HMG-CoA reductase	65,000	(3)	200,000
Acute-phase* Reactants			
Haptoglobulin	40,000	(2)	85,000
α_1-acid glycoprotein	-		43,000
aspartate aminotransferase	50,000	(2)	100,000
C-reactive protein	23,000	(6)	140,000
α_1-antitrypsin	-		44,100
caruloplasmin	53,000	(2)	124,000
α_2-glycoprotein	-		49,000

*from various sources.

taken from Fasman, 1976.

sedimenting in the 28S regions of the gradient where the increase is observed with adaptive synthesis.

A further discussion of variations in poly(A)-mRNA levels is included in the discussion on hormonal variations.

1.4 Conclusions: The Mechanism of Adaptive Synthesis and the Effect of Inflammation.

The mechanism of hepatic adaptive synthesis, and the influence of inflammation upon it, appears very complex. It involves a number of interrelated factors which attempt to coordinate cellular metabolism while adapting it to the impetus of two external influences. The two processes of adaptive synthesis and inflammation do not occur antagonistically but rather influence the cellular organization so as to ensure survival of the whole animal over a long-term. The difficulty in comparing the processes and attempting to describe a mechanism are enhanced by the nature of the two processes. One, inflammation involves activation from a single site at some distance in the body from the liver and entails production and secretion of proteins in response to this activation. The other, adaptive synthesis, involves activation of the whole animal in response to the stimulus of refeeding and entails an internalized response inside the liver.

The effects of both adaptive synthesis and inflammation involve changes in the activity and amounts of fatty acid synthetase present implicating the importance of long-term control mechanisms. Short-term mechanisms although important in the control of lipogenesis do not appear to affect FAS activity.

These long-term mechanisms appear to involve both specific and general controls on FAS degradation. Adaptive synthesis resulted in a decrease in FAS degradation while inflammatiion enhanced the

degradation of the enzyme prior to changes in the rate of synthesis being observed. This indicates the presence of two degradative mechanisms.

Changes in the relative rates of FAS synthesis were also observed for both the enzyme "overshoot" with adaptive synthesis and the suppression of this overshoot by inflammation.

Taking into account both the variation in degradation and synthesis observed, the variations in FAS levels can largely be accounted for. The mechanisms controlling protein synthesis during adaptive synthesis and inflammation involve both transcriptional and translational changes. Changes in the levels of poly(A)-containing messenger RNA can only partially account for the increased synthesis indicating that translational controls are involved. The evidence indicates, that at least partially, some of the translational control resides in the transcribed message. The signal-controlling ribosome-membrane association is known to be part of the message. Variations in the size of mRNA produced and the possibility of size-dependent rates of translation are also related to transcriptional changes. The evidence presented here also indicates that translational controls are involved in activation of cellular messanger RNA with refeeding, after a period of starvation.

2. EFFECT OF INFLAMMATION UPON HORMONAL CHANGES DURING ADAPTIVE SYNTHESIS.

A variety of reports dealing with the possible roles of hormones in the regulation of FAS levels in rat tissues have appeared. For example, diabetic rats have severely depressed levels of hepatic FAS which can be restored to normal or near normal levels by administration of insulin (Burton *et al.*, 1969; Craig *et al.*, 1972; Volpe and Marasa, 1975), or by adrenalectomy (Volpe and Marasa, 1975), indicating possible regulatory roles for insulin and cortisol. The effects of these hormones may be indirect or secondary effects, as rats fed a high fructose diet exhibit elevated FAS levels together with normal or subnormal serum insulin levels (Volpe and Vagelos, 1974; Kumar *et al.*, 1977), and injection of cortisol had no effect on hepatic FAS levels, although in adipose tissue FAS synthesis was inhibited (Volpe and Marasa, 1975). More recent studies using liver explants have found an obligatory role for insulin and supportive roles for triiodothyronine and cortisol in hepatic FAS induction (Joshi and Aranda, 1974a) and in stearoyl-CoA desaturase expression (Joshi and Aranda, 1979b).

In previous studies the response of FAS levels to administered hormones was monitored in rats under a variety of physiological and nutritional stresses. In our experiments, serum or hepatic levels of insulin, cortisol, glucose, cAMP and glycogen were monitored in rats which were subjected to experimental inflammation and/or which were synthesizing FAS in response to fat-free feeding following starvation.

Liver glycogen levels increase rapidly during fat-free feeding (Fig. 52; Shutler *et al.*, 1977) and induction of inflammation caused

immediate and rapid glycogenolysis, and elevated serum cortisol (Fig. 57). The rise in cortisol was unexpected as glucocorticoids are gluconeogenic and cause increased glycogen synthesis in liver (Geelen *et al.*, 1978). Shikama *et al.* (1980) have demonstrated a drop in glycogen and an increase in glycogenic activity occurring as fasting in rats developed for a longer time.

Fat-free feeding following starvation caused a rapid increase in serum glucose levels (Fig. 55), which declined and leveled off within 12 h, before falling again after 48 h of refeeding. Serum insulin levels showed an increase over the 12 h following refeeding, but the increase was small and of doubtful significance (Fig. 56). Other studies in the rat (Gibson *et al.*, 1972) have shown much larger relative increases in serum insulin concomitant with the adaptive synthesis of FAS. The reason for this discrepancy is not clear, but could be related to differences in methods used for insulin assay, particularly when the values reported for serum insulin by Gibson *et al.* (1972) were much lower in starved, normal and refed rats than the levels we have obtained by radioimmune assay. Fig. 56 also shows a marked increase in serum insulin 48 h after the start of refeeding, which may reflect a levelling off of glycogen storage in the liver (see Fig. 52). Other studies have indicated insulin may not play an indispensible role in hepatic glycogenesis in the fed and early refed states (Shikama and Ui, 1978; Shikama *et al.*, 1980).

Serum cortisol levels were essentially unaffected by starvation and refeeding (Fig. 57), while hepatic cAMP levels were markedly depressed under the same conditions (Fig. 54). The latter observation is consistent with the findings that glucagon, cAMP and theophylline suppress hepatic synthesis of FAS (Volpe and Marasa, 1975; Lakshaman

et al., 1972).

The effects of experimental inflammation on serum glucose, insulin and cortisol are presented in the inserts of Figures 55, 56 and 57 respectively. Serum glucose was rapidly elevated, peaking at around 12 h after inflammation, perhaps because of the glycogenolysis caused by inflammation (Shutler *et al.*, 1977). Serum insulin was also elevated by inflammation, but the peak values were seen at 24 h which is 12 h after the peak in serum glucose levels. The reason for this protracted lag is not known. Kaminski *et al.* (1979) have recently reported that rats subjected to inflammatory stress by bacterial infection exhibit elevated serum insulin levels after 48 h infection. Neufeld *et al.* (1980) observed an immediate rise in serum insulin levels when fasted rats were subjected to bacterial infection. They failed to observe this effect in hypophysectomized rats whereas removal of the thyroid, adrenal or gonodal hormones (through organ removal) had no observed effect. Inflammation also caused elevated serum cortisol levels (Fig. 57). This 3-4 fold increase peaked about 12 h after inflammation, and is consistent with the known anti-inflammatory role of glucocorticoids (Dannenberg, 1979).

Serum glucose levels in rats subjected to experimental inflammation after the onset of adaptive synthesis of FAS were elevated above the levels characteristic of adaptive synthesis or inflammation alone (Fig. 55), which may result from the glycogenolytic effects mentioned previously (Shutler *et al.*, 1977). There was variability-possibly oscillation - in the glucose levels following inflammation 6 h or 8 h after the onset of refeeding, but the levels remained generally higher than without inflammation. Under the same conditions, serum insulin levels increased (Fig. 56) and varied similarly to glucose

levels, but the changes were delayed by around 12 h. Cortisol levels were elevated rapidly, peaking within 12 h, in rats subjected to inflammation 6 h or 8 h after the start of fat-free diet refeeding (Fig. 57). The effect was essentially identical to the effect of inflammation uncomplicated by starvation and refeeding (inset Fig. 57). The decline in hepatic cAMP levels seen during adaptive synthesis was rapidly reversed by the induction of inflammation (Fig. 54), particularly with relatively short-time intervals between refeeding and inflammation.

These results support the notion that insulin has an indirect rather than a direct role in the control of FAS levels (Volpe and Vagelos, 1974; Kumar *et al.*, 1977), as serum insulin did not increase dramatically during the most rapid phase of adaptive synthesis (Fig. 52), and more particularly since insulin levels were elevated by inflammation at the same time as increased FAS activity was suppressed. The complexity of the hormonal situation increases when considering proposals for short-term control of lipogenesis by insulin and glucagon (Beynen *et al.*, 1979), different roles for insulin and glucagon at different locations throughout the body (i.e. impaired granulocyte adherence with inflammation during diabetes) (Bagdade and Walters, 1980), and suggestions that both neural and hormonal impulses from the hypophysis are involved in stimulation of insulin and glucagon release from the pancreas (see Neufeld *et al.*, 1980).

Serum cortisol and hepatic cAMP levels changed rapidly in response to inflammation (Fig. 57 and 54), perhaps indicating a more direct role in the regulation of FAS levels in liver. Cortisol levels were unchanged during adaptive synthesis of FAS, arguing against a direct role for this hormone in stimulating FAS synthesis,

inflammation caused a marked increase in serum cortisol concomitant with a decline in hepatic FAS levels, indicating a possible role in suppressing FAS synthesis or increasing degradation. This interpretation is consistent with the suggestion of Volpe and Marasa (1975) that the stimulation of FAS synthesis by insulin is counteracted by inhibition due to glucocorticoids.

Mechanistically the hormonal effects appear to be mediated at a number of different levels and involve both influences on protein synthesis and degradation. Glucagon is believed to cause an inhibition of general protein synthesis and an increase in degradation. mediated by different mechanisms in the rat liver (Ayuso-Parrilla *et al.*, 1976). Pfeifer (1978) proposed that insulin blocks autophagic degradation possibly by inhibiting the formation of new autophagic vacuoles. Wilde *et al.* (1980) found that adrenalin partially inhibited the degradative increases in FAS and other cytosolic proteins in mammary gland cultures. They also found that addition of both insulin and adrenalin completely blocked the degradative increases leading to the proposal that adrenalin acted upon the non-lysosomal component of the degradative system. More recently Hopgood *et al.* (1980) have proposed that glucagon acts via cAMP to increase lysosomal degradation while insulin inhibited this process by a mechanism independent of cAMP. Evidence has also implicated cAMP increases in decreasing the levels of messenger RNA for fatty acid synthetase and other lipogenic enzymes (Spiegelman and Green, 1981). Pry and Porter (1981) found that glucagon and cAMP caused a 3-fold reduction in the levels of mRNA translating for FAS in the livers of rats refed a fat-free diet for 12 h. They also found an 11-fold decrease in FAS mRNA levels in diabetic rats as compared to similar

animals treated with insulin. Other examples of hormonal controls over messenger RNA levels include both general and specific induction of tyrosine aminotransferase by different hormones (see Hill *et al.*, 1981). A number of hormonally-induced variations in mRNA levels for secretable proteins have also been reported including albumin (Peavy *et al.*, 1978) and α_{2u}-globulin (Roy *et al.*, 1980).

2.1 Conclusions: Hormonal Involvement in the Control of Adaptive Synthesis and the Inflammatory Response.

Hormonal involvement in the control of adaptive synthesis and the inflammatory response is extremely complex and has not been clearly elucidated. The hormonal responses to inflammation and adaptive synthesis involves interrelated effects occurring at various locations throughout the body and the different levels of cellular control of hepatic metabolism. The use of mammalian cell culture in studying the responsiveness of individual cell types to hormonal and environmental stresses may provide a vehicle for overcoming the difficulties involved in discerning hormonal effects in a whole animal situation.

3. EFFECTS OF INFLAMMATION ON OTHER ASPECTS OF LIPOGENESIS DURING ADAPTIVE SYNTHESIS.

Preliminary studies were done on some other aspects of lipogenesis. The effects of inflammation on the cholesterol and triglyceride levels in the liver during adaptive synthesis and on two other enzymes involved in lipogenesis were investigated.

As expected from observation of the variations in FAS with refeeding and inflammation, triglyceride levels increased much more in non-inflamed, refed animals than in inflamed animals (Fig. 58). Starvation for 48 h resulted in a large decline in hepatic triglyceride levels (40%) which were increased to above normal levels by 24 h of refeeding. Inflammation depressed this increase by 50%. The total cellular lipid decreased only slightly with starvation and increased only 12% with 24 h of refeeding. Inflammation at 0 h of refeeding enhanced the initial increase in lipid levels by 25% after 12 h of refeeding but this decreased to the same level as non-inflamed animals by 24 h of refeeding. This suggested the influence of membrane lipid components as inflammation is known to increase the levels of membrane components (see Historical). Figure 57 indicates that cholesterol, a principal lipid membrane component, decreased slightly with starvation and increased dramatically with refeeding and inflammation. Previous evidence indicated that variations in cholesterol were due to changes in the rates of synthesis and degradation of the principal enzyme involved in cholesterol synthesis, HMG-CoA reductase (for review see Bortz, 1973). More recently dietary increases have been found which alter the levels of HMG-CoA reductase while hepatic lipid levels remained unchanged (Marlett *et al.*, 1981). This suggests the existence of further complexities in the control of

lipogenesis, as the additional cholesterol was excreted directly or as bile acids.

Comparison of the variations of acetyl-CoA carboxylase (Fig. 59) and ATP-citrate lyase (Fig. 60) with adaptive synthesis and inflammation indicate ATP-citrate lyase was suppressed in a manner similar to that observed for FAS while the suppression of acetyl-CoA carboxylase was to a much lesser degree.

ATP-citrate lyase activity increased 25-fold with starvation and refeeding which is similar to the results from earlier studies (Inoue *et al.*, 1966). Inflammation reduced the increase by 85% implying control is exerted at the level of acetyl-CoA supply. Early evidence indicated the activity was dependent upon the nutritional and hormonal status of the animal. These studies demonstrated that the rate of enzyme synthesis varied coordinately with other lipogenic enzymes, although ATP-citrate lyase levels varied after the variation was observed in fatty acid synthesis suggesting it does not play a major regulatory role (see Van Golde and Ven Den Bergh, 1977).

Acetyl-CoA carboxylase levels increased 15-fold with starvation and refeeding and this increase was suppressed 25% by inflammation. Previous evidence indicates that acetyl-CoA carboxylase levels undergo long-term changes in response to nutritional and hormonal factors (see Volpe and Vagelos, 1976). The variations in hepatic lipogenic enzymes usually occur coordinately but there are a number of exceptions to this rule. The increases in fatty acid synthetase and ATP-citrate lyase observed with refeeding and the suppression of these increases by inflammation were very similar (Fig. 31 and Fig. 60), while the

suppression of acetyl-CoA carboxylase levels was much less. Diamant and Shafrir (1975) found that glucocorticoid administration to rats led to a marked increase in hepatic acetyl-CoA carboxylase while FAS increased to a lesser extent over a longer period time and ATP-citrate lyase levels didn't appreciably increase. The authors propose that the rise in lipogenic capacity, through a selective increase in acetyl-CoA carboxylase may be the cellular mechanism of restraining the ketogenesis from acetyl-CoA despite the increased fat utilization during glucocorticoid excess. Other studies with hyperinsulinemia in fetal rhesus monkeys found similar increases in hepatic FAS and ATP-citrate lyase levels (McCormick *et al.*, 1979), while Philipp and Shapiro (1981) found estrogen stimulation of *Xenopus laevis* involved increased HMG-CoA reductase and acetyl-CoA carboxylase but no change in FAS levels. Goodridge (1973) found if neonatal chicks are not fed, hepatic acetyl-CoA carboxylase activity remained unchanged but FAS levels increased. Also if eggs are incubated in 100% oxygen for 24 h hepatic FAS activity increased while acetyl-CoA activity remained unchanged.

This variation in the inflammatory suppression of the three lipogenic enzymes during refeeding must be dependent upon the nature of the processes stimulated. The large suppression of ATP-citrate lyase was unexpected as the acetyl-CoA produced is utilized in both the responses to refeeding and inflammation. It may involve the suppression of ketonemia observed in infected, starved animals (Neufeld *et al.*, 1980) and with glucocorticoid stimulation of acetyl-CoA carboxylase and not ATP-citrate lyase discussed previously.

3.2 Conclusions: Specificity of the Inflammatory Effects on Adaptive Synthesis.

Volpe and Marasa (1978) concluded that FAS was more important in the long-term regulation of fatty acid synthesis than acetyl-CoA carboxylase. In our experiments acetyl-CoA carboxylase levels showed little sensitivity to the effects of inflammation which agrees with the previous conclusions. Inflammation must also exert its influence at other locations in hepatic metabolism, as both the levels of cholesterol and ATP-citrate lyase respond to inflammation.

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