REGULATION OF VOLATILE FATTY ACID UPTAKE BY BOVINE HEART, LIVER AND KIDNEY TISSUE

By

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ABSTRACT

REGULATION OF VOLATILE FATTY ACID UPTAKE BY BOVINE HEART, LIVER AND KIDNEY TISSUE

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Control of volatile fatty acid uptake by bovine heart, kidney and liver tissue was studied by purification and characterization of acyl CoA synthetases, enzyme responsible for conversion of the freely permeable fatty acids to the corresponding impermeable coenzyme A derivatives. Tissue from lactating Holstein cows was used. A second experiment was designed to test whether these enzymes were present at birth or developed in response to rumen production of volatile fatty acids. Bull calves were weaned as soon as possible and fed a hay/corn ration. Animals were slaughtered at -14,0,1,7,14,40,60, and 120 days of age. An additional group of calves was maintained on an all milk diet and slaughtered at 60 and 120 days of age. Enzyme activity in mitochondrial and cytosolic fractions of heart, kidney and liver tissue was monitored using acetate, propionate, butyrate, and valerate as substrates. Plasma acetate concentration in peripheral blood was determined at slaughter.

Bovine heart mitochondria contained predominantly one volatile fatty acid activating enzyme, acetyl CoA synthetase, which activated acetate, propionate and acrylate. The Michaelis-Menten constant for acetate was $2x10^{-4}M$. The enzyme was a glycoprotein, composed of one polypeptide chain of apparent molecular weight 67,500. Significant enzyme activity was present in the fetus, increased with age and was not influenced by diet. Cytosolic acetyl CoA synthetase was also present at birth, increased with age but was significantly lower ($P < .05$) in animals in which rumen development had been delayed by feeding an all milk diet. Cytosolic but not mitochondrial acetate activating was correlated with blood acetate concentration ($r=.8438$, $P<.01$).

Bovine liver mitochondria contained an enzyme, propionyl CoA synthetase, activating propionate and acrylate, and a butyrate activating fraction with broad substrate specificity for short and medium chain length fatty acids. Based on kinetic studies this fraction is composed of two enzymes; a butyrl CoA synthetase and a valeryl CoA synthetase. The apparent molecular weights of the three liver enzymes were 72,000, 67,000 and 65,000 respectively. The Michaelis-Menten constants of propionyl CoA synthetase for propionate, ATP and coenzyme A were $1.3x10^{-3}M$, $13x10^{-4}M$ and $6.3x10^{-4}M$ respectively. Mitochondrial propionate, butyrate and valerate activation were low at birth and increased as the animal matured. Propionate activation was lower (15 mμmoles/min/mg protein) in

animals fed a liquid diet for 120 days than in animals fed solid feed (27 mμmoles/min/mg protein). Mitochondrial propionate, butyrate and valerate activation were correlated with blood acetate level ($r=.7450, .8034, .7177, P<.05$). Cytosolic activation of these substrates was also low at birth and increased with age but was not influenced by diet or correlated with blood acetate concentrations. Acetyl CoA synthetase activity was negligible in both the mitochondrial and cytosolic fractions of liver tissue.

Bovine kidney mitochondria contained the acetyl CoA synthetase characteristic of heart and the enzymes characteristic of liver. The Michaelis-Menten constant of propionyl CoA synthetase for propionate was 2.54×10^{-3}M. Mitochondrial propionate, butyrate and valerate activation were low at birth and increased significantly with age ($P<.05$). Animals maintained on a liquid diet for 60 and 120 days of age had significantly lower fatty acid activating ability ($P<.05$) than animals fed solid feed for the same number of days. Mitochondrial propionate, butyrate and valerate activation were correlated with blood acetate concentration ($r=.7356, .6966, .6327, P<.05$). Cytosolic activation of these substrates was also low at birth and increased with age irrespective of dietary regime.

Volatile fatty acid uptake by ruminant tissues is regulated by different acyl CoA synthetases with overlapping substrate specificities.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
AMP	adenosine monophosphate
Tris	tris (hydroxymethyl) amino methane
TCA	trichloroacetic acid
EDTA	ethylene deamine tetraacetic acid
TEMED	tetra methyl ethylene diamine
Bis	methylenebisacrylamide
DEAE	diethylaminoethyl

INTRODUCTION

In ruminant animals the microbial fermentation of dietary carbohydrate in the rumen leads to the production of large quantities of volatile fatty acids, principally acetate, propionate and butyrate. Very little dietary hexose is therefore available for absorption from the gastro-intestinal tract and the animal is critically dependent on gluconeogenesis for the provision of glucose in both the fed and fasted state.

The ruminant animal has evolved a unique system of metabolism which allows it to spare glucose for essential body functions and to use the ruminally derived fatty acids as alternate substrates for both energy generation and storage. In addition up to 50% of the total glucose requirement of the animal can be met by synthesis from propionate, a process which occurs in liver. In contrast, the newborn ruminant has a system of metabolim based on glucose, comparable to that of the monogastric animal.

Volatile fatty acids are freely permeable to cellular membranes. In order for uptake and subsequent tissue metabolism they must first be trapped within a particular cellular compartment by conversion into the impermeable coenzyme A derivative. This so-called activation reaction

is catalyzed by a series of enzymes termed acyl CoA synthetases.

Studies with tissue homogenates have demonstrated that different tissues can activate different volatile fatty acids. For example, liver tissue can activate propionate, butyrate, and valerate but not acetate, heart tissue can activate acetate and propionate and kidney tissue can activate all these substrates.

It was the purpose of this work to elucidate why different tissues had different patterns of volatile fatty acid activation and to try and relate this to the physiological function of the tissue. The approach was to purify the fatty acid activating enzymes from the different tissues and to perform simple kinetic studies to determine how enzyme activity and thus volatile fatty acid uptake might be controlled. Liver, kidney and heart mitochondrial tissue were chosen since these tissues show very different patterns of volatile fatty acid activation and because the physiological function of these organs is so different. Since gluconeogenesis from propionate is so important especially in dairy cows producing large quantities of milk, a primary objective was to try and isolate a distinct propionyl CoA synthetase in the hope that if such a enzyme existed and if enzyme activity could be modulated in some way then many of the problems facing these animals could be alleviated.

A second objective was to determine if these enzymes were present at birth or whether they developed as the shifts in metabolism from the pre-ruminant to the adult ruminant form occurred.

The broad objective of this research was to identify ways in which tissue uptake of volatile fatty acids could be controlled so that feed efficiency and milk production could potentially be improved and the incidence of metabolic diseases such as ketosis minimized.

LITERATURE REVIEW

Many of the patterns of intermediary metabolism that occur in different animal species can be related to the evolutionary adaptations which have occurred in the digestive tract of these animals. Monogastric animals have a system of intermediary metabolism based upon glucose which is derived from dietary carbohydrates. This glucose is used as a source of energy and carbon for tissue metabolism. In ruminant animals very little dietary hexose is available for absorption from the gastro-intestinal tract because of the microbial fermentation of dietary carbohydrate to volatile fatty acids which occurs in the rumen.

As a result of the fermentation process ruminant animals are critically dependent on the process of gluconeogenesis for provision of glucose at all times (Bergman,]973; Young,]977). So that glucose levels can be maintained at adequate levels for critical body functions these animals have developed a pattern of intermediary metabolism which allows them, when possible, to utilize volatile fatty acids, primarily acetate, as alternative substrates to glucose for oxidative and synthetic purposes and to synthesize large quantities of glucose from ruminally derived propionate. Thus the volatile fatty acids as well as

glucose are major metabolic substrates in these animals.

The manner in which a particular volatile fatty acid can influence carbohydrate and/or lipid metabolism depends on a number of factors; whether a particular acid is presented to a given organ or tissue; whether that organ or tissue has the capability to take up and incorporate that acid into its metabolic machinery; and lastly the use to which a given acid will be put will depend on the particular organ or tissue involved and the physiological and nutritional status of the whole animal at that time.

This review will consider five topics: (1) quantities and type of volatile fatty acid available to the different tissues; (2) enzymes responsible for the trapping of these acids within a particular cellular compartment; (3) biochemical pathways in which these acids can be metabolized; (4) the role of volatile fatty acids in the intermediary metabolism of key organs such as liver, kidney, mammary gland and adipose tissue and (5) development of the ruminant type of metabolism from the pre-ruminant type of metabolism of the newborn ruminant will be described. For clarity pathways of carbohydrate and lipid metabolism in the non-ruminant will be described also and the differences between the ruminant and non-ruminant patterns of intermediary metabolism emphasized.

Availability of Volatile Fatty Acids to the Various Tissues

All water-soluble metabolites are absorbed from the gastro-intestinal tract directly into the hepatic portal blood system. These metabolites can then be subjected to further metabolism in the liver before release to the general body circulation. Information on the availability of volatile fatty acids to the various tissues has been obtained by studies with whole animals in which rumen, hepatic portal, and peripheral blood levels have been determined (Annison et al., 1957; Cook and Miller, 1965; Bergman, 1974; Baird et al., 1975). These investigators working with sheep, goats, and lactating cows have shown that acetate, propionate, and butyrate are present in rumen fluid and that the molar proportions of these acids depends on diet. For a high roughage diet the molar proportions would be 70:20:10 whereas a high grain ration would yield acetate, propionate, and buyrate in the ratio 45:45:10; on restricted roughage diets there is increased propionic acid production in the rumen. Although compared to monogastric animals there is a relatively constant production of metabolites from the gastro-intestinal tract as a consequence of the presence of the rumen microbiota, rumen volatile fatty acid levels do increase after feeding (Baird et al., 1975; Chase et al., 1977).

Rumen epithelial tissue converts butyrate to β-hydroxybutyrate (Pennington and Sutherland, 1954; Ramsey and Davis, 1965) thus butyrate is not present in

significant amounts in rumen vein or portal blood (Cook and Miller,]965) and thus is not made available to either liver or peripheral tissues in significant quantities. Bergman (]974) using both in vitro and in vivo techniques estimated that 30-45% of ruminal acetate, 50-65% of ruminal propionate, and 85-90% of the butyrate were metabolized by the rumen wall. Pennington and Sutherland(1954) have shown that some propionate is metabolized to lactate (20-50%). However it is now generally agreed that most propionate and acetate are taken via hepatic portal blood to the liver. Concentrations of hepatic portal propionate in sheep vary depending on the diet (Cook and Miller, 1965; Bergman, 1974) from 0.2-1.3 mM on a high roughage type diet to 0.5-2.0 mM on a high grain ration. Acetate in the hepatic portal vein usually ranges from 1-2 mM and butyrate is usually present at about 0.025 mM (Cook and Miller, 1965). Chase et al. (1977) have shown that total portal concentrations of volatile fatty acids change rapidly after feeding.

Measurements of volatile fatty acid levels in peripheral blood (Annison, 1954; Cook and Miller, 1965; Baird et al.,]975) have established that the acetate concentration is 1-3 mM, the propionate concentration is low 0.02-0.04 mM, and butyrate is approximately 0.02 mM or is undetectable. Ross and Kitts (]973) have demonstrated that peripheral volatile fatty acid levels increase after feeding but not as rapidly as the changes which occur in

portal blood. Plasma acetate levels fall from 0.65 mM in fed steers to 0.2 mM after 2-4 days fasting (Pothoven and Beitz, 1975). On refeeding plasma aetate levels may reach 0.9 mM within 6-8 days.

Hepatic tissues are therefore presented with acetate, propionate, lactate, and β-hydroxybutyrate whereas extra-hepatic tissue receives mainly acetate plus minor quantities of propionate and β-hydroxybutyrate. It must be emphasized that these statements do not imply that a given tissue can always utilize what is presented. This will depend on whether the appropriate enzymes necessary for their uptake are present. This will be discussed in the following section.

Another source of plasma acetate not of direct dietary origin is available. This is acetate produced endogenously probably from the β-oxidation of fatty acids (Annison and White, 1962). This occurs primarily in liver although other tissues such as heart, brain, and skeletal muscle are known to produce endogenous acetate under some conditions.

The observation that acetate is present in the blood of fasted non-herbivores at a concentration of approximately 0.1-0.5 mM (Ballard, 1972) suggested that these animals also produce endogenous acetate since in this case very little acetate could be provided from the diet. The evidence would therefore imply a broader role of acetate in the intermediary metabolism of all mammals than has been previously recognized.

The capacity of ruminant liver to produce endogenous acetate is significantly greater than that of its non-ruminant counterpart (Baird et al., 1974; Costa et al., 1976). Livers of lactating cows (Baird et al., 1974) and ewes (Costa et al., 1976) produce substantial quantities of endogenous acetate as do animals made diabetic by alloxan. Knowles et al. (1974) theorized that this endogenous acetate could be a means of redistributing energy furnishing substrates from adipose via liver to tissues which have the metabolic machinaery to use large quantities of acetate e.g. heart, mammary gland. It seems reasonable to suppose that the greater capacity of ruminants for endogenous acetate production may be related to the fact that these animals have already modified their metabolism so that volatile fatty acids, primarily acetate, can be readily utilized by many peripheral tissues. Endogenous acetate would therefore be a very efficient means of redistributing energy around the animal's body. Although there is some controversy over the exact enzymes responsible for hydrolyzing acetyl CoA to acetate (Costa and Snoswell, 1975; Costa et al., 1976) it is clear that, whatever the mechanism, this process may have profound effects on the intermediary metabolism of mammals.

The current state of our knowledge on the source of both exogenous and endogenous volatile fatty acids in ruminants is presented schematically in Figure 1.

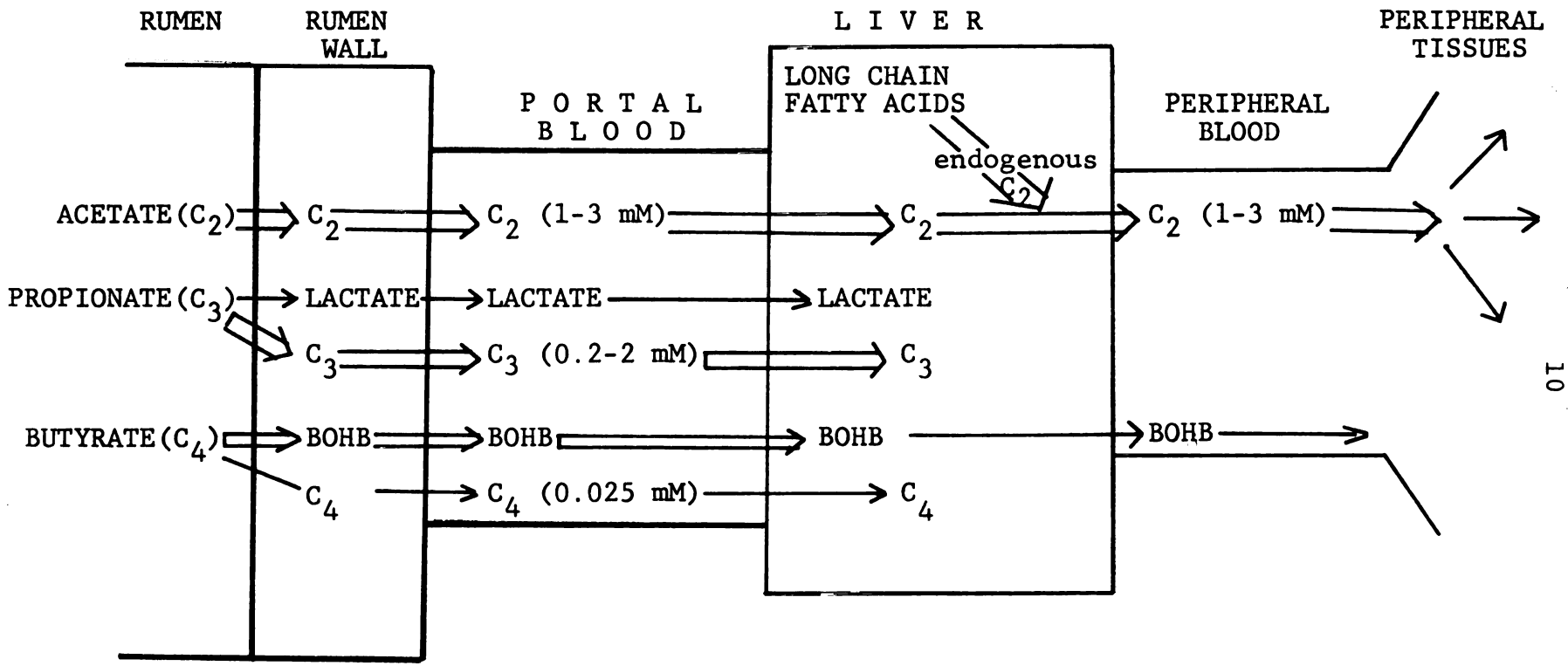


Figure 1 Volatile fatty acids available to the different ruminant tissues.

Uptake of Volatile Fatty Acids by the Various Tissues

Mechanism of uptake

Uptake of a particular volatile fatty acid depends on whether it can be trapped in a given tissue as the coenzyme A derivative - a high energy form. Volatile fatty acids are freely permeable to cellular membranes; however the corresponding acyl CoA derivatives are not (Spencer and Lowenstein, 1962). This trapping process is analagous to the trapping of glucose in a cell as glucose-6-phosphate by hexokinase. The enzymes responsible for conversion to the acyl coenzyme A derivatives are termed the acyl CoA synthetases. These enzymes catalyze a reaction represented by:

(1) VOLATILE FATTY ACID + ATP + CoA-SH $\longrightarrow$ ACYL-AMP + P-P_i

(2) ACYL-AMP + CoA $\longrightarrow$ ACYL-CoA + AMP

This biphasic reaction mechanism was first proposed by Berg (1956) for acetate activation by yeast acetyl CoA synthetase.

Tissue distribution and intracellular localization of acyl CoA synthetases

Acyl CoA synthetases appear to be present in most mammalian tissues. Furthermore, the short and medium chain acyl CoA synthetases are members of a small group of enzymes which are present both in the cytosol and mitochondria (Aas and Bremer, 1968; Aas, 1971; Scholte *et al.*, 1971; Barth *et al.*, 1971; Ballard, 1972; Quraishi

and Cook, 1972). In general (Wada and Morino, 1964) enzymes such as phosphoenolpyruvate carboxykinase, malate dehydrogenase, and aspartate amino transferase, showing a bimodal distribution between both compartments are distinct proteins. Therefore although none of cytoplasmic acyl CoA synthetases have yet been purified due to their instability it is probable that they are distinct from the mitochondrial forms.

The mitochondrial forms must be released by freezing and thawing or sonication before they can be detected by normal assay procedures (Aas, 1971; Scholte et al., 1971; Qureshi and Cook, 1975; Cook et al., 1975).

Estimates of the fatty acid activating activity in tissue homogenates have been made for rats, guinea pigs, and ruminants (Aas and Bremer, 1968; Cook et al., 1969; Aas, 1971; Scholte et al., 1971; Scholte and Groot, 1975). In all species each tissue exhibits a very characteristic pattern of volatile fatty acid activation. Values for a cow 14 days postpartum are given in Table 1.

Groot et al. (1976) concluded that kidney, heart, and skeletal muscles of rat and guinea pig tissue homogenates do not contain cytosolic short chain acyl-CoA synthetases, but that all volatile fatty acid activating ability is present in the mitochondrial matrix. It should be pointed out that most other investigators (Aas, 1971; Murthy and Steiner, 1972) have demonstrated some short chain fatty acid activating ability in the cytosol, although

Table 1 Volatile fatty acid activating ability of tissue homogenates prepared from 2-3 year old Holstein cows slaughtered at 10 days postpartum.

ORGAN	CELL FRACTION	SUBSTRATE [a]			
		ACETATE	PROPIONATE	BUTYRATE	VALERATE
LIVER	Cytosol[b]	303±182	1534±289	901±329	747±243
	Mitochondria[c]	95± 76	602±374	381±198	822±171
KIDNEY	Cytosol[b]	314±141	771±293	370± 66	371±147
	Mitochondria[c]	270± 78	350±137	106± 81	87± 66
HEART	Cytosol[b]	480±248	190±114	34± 48	46± 66
	Mitochondria[c]	31± 15	18± 10	4± 3	2± 2
MAMMARY GLAND	Cytosol[b]	450±107	262±171	86± 74	82± 66
	Mitochondria[c]	199± 75	139± 57	27± 28	25± 28
LUNG	Cytosol[b]	46± 35	58± 22	44± 17	53± 15
	Mitochondria[c]	15± 10	16± 6	9± 4	8± 8
ADIPOSE	undetectable in unfractionated homogenates				

[a] the values represent the average values from 6 animals in units/g wet weight± standard deviation. A unit is equal to 1mμmole of substrate reacting in 1 minute.

[b] tissues were homogenized in 0.13 M KCl pH 8.0 containing 10% glycerol and 2.5 mM 2-mercaptoethanol

[c] enzyme activity was liberated from the mitochondria by sonication.

this is generally small relative to the amount found in the mitochondrion. In contrast ruminants have a greater proportion of the volatile fatty acid activating ability in the cytosol (Table 1). In ruminants where there are large quantities of volatile fatty acids produced in the rumen a high cytosolic activation may be a means of trapping these acids against a concentration gradient.

Non-ruminant animals are characterized by significant acetate activating ability in both the mitochondrial and cytosolic fractions of liver tissue (Ballard, 1972; Scholte and Groot, 1975). The ruminant animal however possesses only marginal acetate activating ability in these compartments (Table 1). The presence or absence of a cytosolic acetate activating enzyme may be related to the lipogenic capacity of the liver. Characteristically, lipogenesis occurs primarily in liver in monogastric animals and primarily in adipose tissue in ruminants (Ingle et al., 1972a; 1972b). Bauman (1978) suggested that the site of lipogenesis may be related to the level of volatile fatty acids produced in the gastro-intestinal tract and this in turn would influence the location of the cytosolic acetate activating enzyme. For example in animals with a cecal fermentation such as rabbits and guinea pigs some lipogenesis would occur in both liver and adipose sites and in guinea pigs there is some cytsolic acetate activation although this is lower than that found in a rat (Scholte and Groot, 1975). In ruminants where large quantities of volatile fatty acids

are produced in the rumen lipogenesis has been shifted to the adipose depots with concurrent loss of the cytosolic acetate activating enzyme in liver tissue. The physiological significance of this shift will be discussed in the next section. The reason for a lack of mitochondrial acetate activation in the ruminant liver tissue is not understood at present. Some possibilities will be outlined in the discussion section.

The volatile fatty acid activating ability of the various tissues of the rat, sheep, guinea pig, and cow do not appear to differ substantially (Ballard, 1972; Scholte and Groot, 1975; Table 1). This observation in conjunction with the fact that peripheral blood acetate levels are only 1-2 orders of magnitude less in the non-ruminant than the ruminant has led a number of investigators to postulate that no specific adaptation has occurred in the ruminant to allow it to utilize large amounts of ruminal acetate (Ballard, 1972; Groot et al., 1976). This conclusion may not, however, be valid. Many investigators using the same experimental animal obtain widely varying values of volatile fatty acid activation. Moreover complete data on both a ruminant and non-ruminant speices by the same investigator is not available. Therefore direct comparisons between animals are difficult.

Classification of acyl CoA synthetases

Until recently, based upon purification studies on the mitochondrial forms of these enzymes, it was generally accepted that there were three acyl CoA synthetases. Acetyl

CoA synthetase (EC.6.2.1.1) has been purified from beef heart mitochondria by Campagnari and Webster (1973),from goat mammary mitochondria by Cook et al.(1975) and from bovine mammary mitochondria by Qureshi and Cook (1975) active on both acetate, propionate and acrylate. A butyrl CoA synthetase (EC.6.2.1.2) has been purified by Mahler et al. (1953) from beef liver mitochondria and by Groot (1976) from guinea pig liver mitochondria active on C_4-C_{12} saturated straight chain fatty acids. Similar preparations have been obtained from pig kidney and rabbit liver (Kellerman, 1958), from pig liver particles (Jencks and Lipmann, 1957), human liver and kidney (Moldave and Meister, 1957) and rat liver (Lehninger and Greville, 1953) suggesting the enzyme is common to the liver and perhaps kidney of many species. A long chain acyl CoA synthetase (EC.6.2.1.3) has been partially purified from rat liver microsomes (Bar-Tana et al., 1971) and active on saturated and unsaturated fatty acids C_{12}-C_{18}.

This classification has, however, proved to be too simplistic. As early as 1964 Cook et al. based on studies with tissue homogenates, proposed the existence of a distinct propionyl CoA synthetase. This enzyme has been purified from sheep liver mitochondria by Latimer (1967) and from guinea pig liver mitochondria by Groot (1976). The enzyme exhibits a high specificity for propionate (K_m 0.43 mM) and little affinity for other short chain volatile fatty acids. Webster et al. (1965) have purified a butyrate

activating enzyme from bovine heart mitochondria. This enzyme is different from the butyrl CoA synthetase purified by Mahler et al. (1953) from beef liver. Groot (1976) using guinea pig mitochondria and Killenberg et al. (1971) using beef liver mitochondria have isolated a salicylate enzyme exhibiting maximal activation towards hexanoate and benzoate. This enzyme also activates butyrate. The short chain volatile fatty acid activation in guinea pig mitochondria would be a consequence of activation by three enzymes: one directed towards propionate activation, propionyl CoA synthetase whereas butyrate activation would be due to the presence of two enzymes, the "Mahler" enzyme, and the salicyclate enzyme. This would probably also be true for bovine liver although no distinct propionate enzyme has been isolated from this source.

Groot et al. (1976) and Londesborough and Webster (1974) have published reviews on the molecular and enzymatic properties of the fatty acyl CoA synthetases. Groot has recommended the name butyrl CoA synthetase (EC.6.2.1.2) be reserved only for enzymes with properties identical to those of the enzyme purified from bovine heart mitochondria by Webster et al. (1965) and that the term medium chain acyl CoA synthetase be used for the so-called classical butyrl CoA synthetase as purified by Mahler et al (1953).

A summary of the purification procedures that have been used to isolate the short chain acyl CoA synthetases from mammalian tissues is presented in Table 2.

Table 2 Procedures used to purify acyl CoA synthetased from mammalian sources.

ENZYME	SOURCE	REFERENCE	PROCEDURES
acetyl CoA synthetase	bovine heart mitochondria	Campagnari & Webster 1963	$(NH_2)_4SO_4$ fractionation, chromatography on Sephadex, TEAE cellulose, Sephadex.
	bovine mammary gland mitochondria	Qureshi & Cook 1975	$(NH_2)_4SO_4$ fractionation, chromatography on DEAE-23 cellulose, DEAE-52 cellulose, $CaPO_4$ gel.
propionyl CoA synthetase	guinea pig liver mitochondria	Groot 1976	$(NH_2)_4SO_4$ fractionation, chromatography on phosphocellulose, DEAE-Sephadex.
	sheep liver mitochondria	Latimer 1967	Acetone powder extraction, $(NH_2)_4SO_4$ fractionation, Sephadex chromatography 3x, electrophoresis.
butyrl CoA synthetase	bovine heart mitochondria	Webster et al. 1965	$(NH_2)_4SO_4$ and alumina gel fractionation, chromatography on Sephadex 2x, TEAE-cellulose.
Medium chain acyl CoA synthetase	bovine liver mitochondria	Mahler & Wakil 1953	acetone powder extraction, $(NH_2)_4SO_4$ fractionation 2x, alumina gel fractionation, $(NH_2)_4SO_4$ fractionation

Biochemical Pathways of Volatile Fatty Acid Metabolism

Mitochondrial pathways

Mitochondrial activation of acetate or butyrate and subsequent oxidative degradation via the tricarboxylic acid cycle (TCA cycle) would yield energy in the form of ATP as well as intermediates required for synthetic purposes. Incorporation at the level of the mitochondrion allows the cell to by-pass glycolysis (Ballard et al., 1969; Bauman and Davis, 1974; Bauman, 1976) a process which uses glucose. In ruminants a large proportion of energy is derived by the peripheral tissues from oxidation of acetate and this spares the utilization of glucose for other more essential purposes such as for the brain, for the fetus during pregnancy, as a precursor of glycerol for lipid synthesis, and as a precursor for the milk sugar lactose (Young, 1977).

Only volatile fatty acids containing an uneven number of carbon atoms can give a net synthesis of glucose. Activation of propionate within the mitochondrial matrix and incorporation in the TCA cycle at the level of succinate will yield glucose. This process is restricted to organs, namely liver and kidney, which possess the necessary gluconeogenic enzymes. Mammary tissue even though it has a high requirement for glucose in the lactating state cannot utilize propionate for glucose synthesis (Scott et al.,1976).

Cytosolic pathways

Cytosolic activation of acetate and/or butyrate will yield substrates for lipogenesis (Bauman and Davis, 1974)

and for cholesterol biosynthesis. In ruminants fatty acid synthesis will occur primarily in adipose tissue depots and in mammary tissue when milk fat is being secreted (Ingle et al., 1972a, 1972b; Bauman and Davis, 1974). In monogastric species the site of a active lipogenesis varies but in most cases occurs primarily in liver (Inlge et al., 1972a, 1972b). These investigators have suggested that the shifting of lipogenesis from liver to adipose sites in ruminants has occurred so that gluconeogenesis, a process on which the ruminant animal is critically dependent for a supply of glucose, can occur at all times. Lipogenesis and gluconeogenesis are both processes which compete for energy and carbon skeletons. However the metabolic controls exerted on these pathways preclude them from occurring simultaneously in the same tissue (Tepperman and Tepperman, 1970).

Cytosolic activation of propionate could lead to the synthesis of uneven chain length fatty acids, however, such acids are rare in mammalian systems; most being of even carbon chain length. The physiological basis for propionic acid activation in the cytosol remains to be established although propionate activation in this compartment could be a means of trapping all the propionate available to the cell and then transferring it via the carnitine system to the mitochondrion. This might also be true for the cytosolic activation of acetate particulary in tissues which are not actively synthesizing lipid. The primary uses,

to which the volatile fatty acids are put, in heart, kidney, liver and adipose tissue are shown in Figure 2.

Role of Volatile Fatty Acids in the Intermediary Metabolism of Different Organs and Tissues

Liver metabolism

General - The liver plays a major role in the control of intermediary metabolism of all mammal by integrating and coordinating the metabolism of the whole body. Most of the incoming dietary nutrients pass via the hepatic portal blood system to the liver where they are package according to the specific requirements of the animal at that time and then either stored or distributed to the peripheral tissues. Most of the energy required for liver function is obtained by oxidation of amino acids and fatty acids. In ruminants, however, propionate can be oxidized to carbon dioxide (Hood et al., 1972) and this rate is five times greater than that of acetate oxidation.

The primary difference between the liver metabolism of ruminants and non-ruminants, is that in the former gluconeogenesis occurs continuously. Less than 10% of the glucose requirement of the ruminant is absorbed from that gastrointestinal tract(Bensadoun et al., 1962; Young, 1977). Therefore ruminant animals are dependent on the continual process of gluconeogenesis to provide the remaining 90%. If the gluconeogenic process breaks down then severe metabolic disturbances such as ketosis in cattle, pregnancy

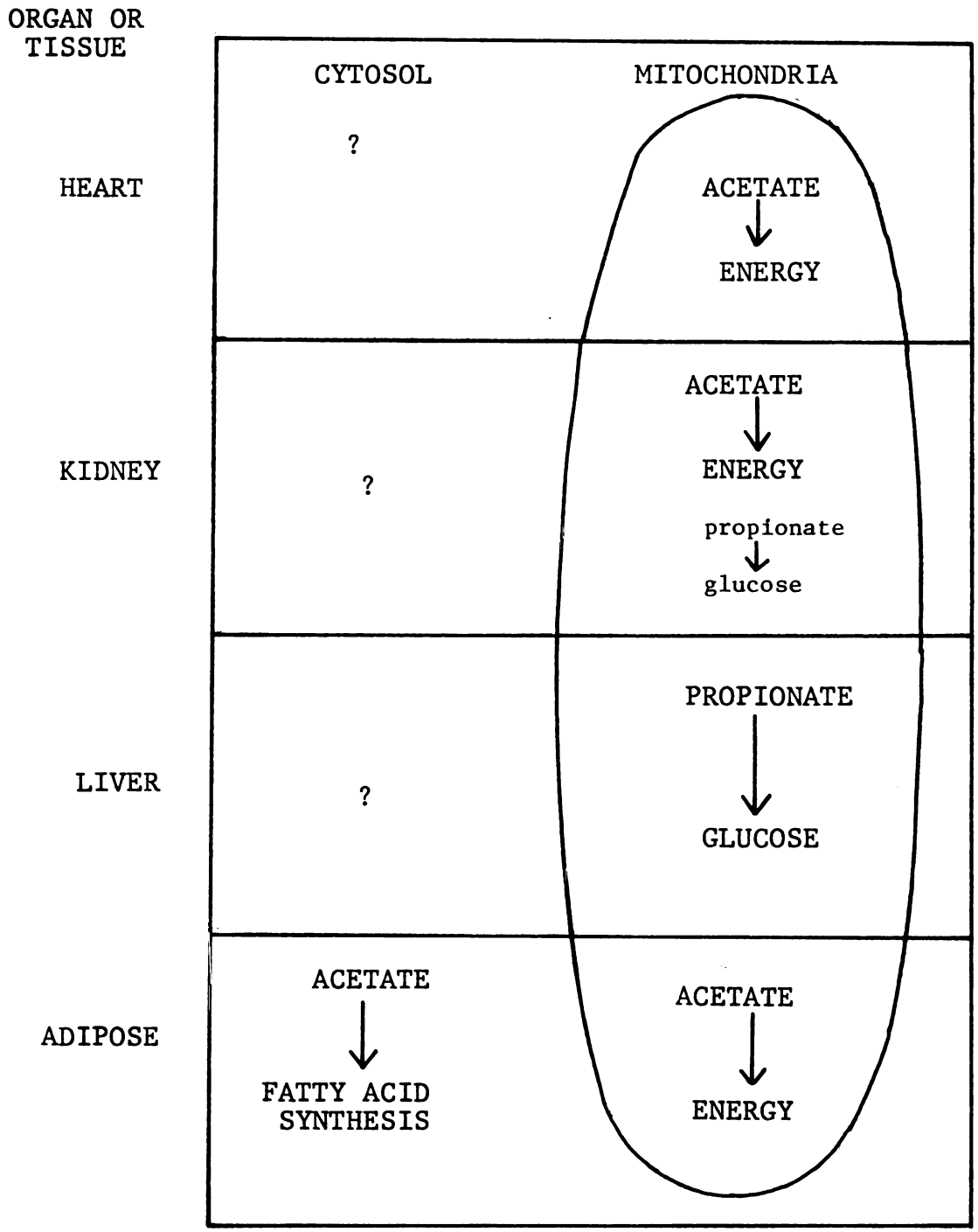


Figure 2 Primary functions of volatile fatty acids in the intermediary metabolism of ruminant heart, kidney, liver and adipose tissue.

toxemia and twin lamb disease in sheep can result (Bergman, 1973; Young, 1977). Gluconeogenesis in ruminants has been reviewed by Leng (1970), Bergman (1973), and Young (1977).

Relationship of gluconeogenesis to physiological and nutrional state - The amount of glucose synthesized in the ruminant liver is dependent on the physiological status of the animal. For example the glucose requirement of a dairy cow producing 89 Kg of milk daily has been calculated to be 7.4 Kg/day (Young, 1977) of which 60% is used for lactose synthesis. Assuming the cow had to synthesize 90% of this then 6.6 Kg of glucose had to be synthesized per day. Steers (160-250 Kg) fed slightly above maintenance still require synthesis of approximately .6 Kg of glucose per day (Young, 1977). Bergman (1973) has summarized the glucose requirements for the normal non-pregnant, pregnant, and lactating sheep as 100, 180, and 320 g/day respectively. These data emphasize the importance of gluconeogenesis as a continual process in ruminants and the tremendous influcence imposed on this process by lactation and pregnancy.

The major gluconeogenic precursors in ruminants are propionate and glucogenic amino acids (Young, 1977). Thus maximal rates of gluconeogenesis occurring in the liver of ruminants have been observed 2-4 hours after feeding, when these precursors are available (Ballard et al., 1969; Katz and Bergman, 1969; Bergman, 1973; Trenkle, 1978). In contrast, maximal rates of gluconeogenesis in non-ruminants,

occur during fasting, when glucogenic amino acids and glycerol are made available by the breakdown of the body tissue itself (Ballard et al., 1969; Young, 1977).

In ruminants, absolute rates of gluconeogenesis do vary with both the type and quantity of dietary nutrients fed (Leng, 1970; Steel and Leng, 1973). This has been directly related to the intake of both digestible energy and crude protein (Reilly and Ford, 1971). Presumably, in the former case increased glucose synthesis is a consequence of an increased microbial propionic acid fermentation in the rumen; an observation which has led to the development of feed additives such as monensin which increase rumen propionate levels. In the latter case an increased gluconeogenesis would result from an increase in availability of glucogenic amino acids.

Volatile fatty acids as carbon sources for gluconeogenesis - Propionate is the only volatile acid produced in the rumen that is a major source of glucose (Bergman et al., 1966; Judson et al., 1968). This is because acetate and butyrate, as well as the even long chain fatty acids (which are most of them), derived from dietary fat or adipose tissue are converted to acetyl CoA. Subsequent metabolism of acetyl CoA via the TCA cycle results in the loss of two carbons as carbon dioxide. No net gain of oxaloacetate can occur and therefore no net synthesis of glucose (Weinman et al., 1957). At one time butyrate was thought to be an important gluconeogenic compound Potter, 1952; Kronfeld, 1957;

Black et al., 1961) since butyrate injections resulted in hyperglycemia. It is now known that this hyperglycemic action of butyrate is mediated by an increase in glycogenolysis (Ash et al., 1964; Phillips et al., 1965) and possibly also by an increased synthesis of oxaloacetate from pyruvate (Black et al., 1966). Butyrl CoA is an allosteric effector of pyruvate carboxylase, the enzyme which converts pyruvate to oxaloacetate.

Propionate conversion into glucose takes place almost entirely in liver since 90% of hepatic portal propionate is removed by liver in a single circulation. Radioisotope techniques have been used to determine rates of propionate incorporation into glucose in liver (Young, 1977). Results of different investigators have been somewhat variable, in part, because propionate production is proportional to feed intake within a given diet (Yost, 1976; Young, 1977) and also because breeds of different sizes and ages have been used (Bergman, 1973). Bergman et al. (1966) calculated that only 27% of the total glucose synthesized in well fed sheep was derived from propionate. Other studies with sheep (Leng et al., 1967; Judson et al., 1968) have shown that 54% of glucose synthesized could come from propionate carbon. On the basis of their results these investigators suggested that considerable amounts of propionate were converted to lactate in the rumen wall. More recent in vitro studies with calves (Weigand et al., 1975) and with ewes (Weekes. 1972) and in vivo studies with ewes

(Weekes and Webster, 1975) have established that significant in vivo conversion of propionate to lactate by rumen epithelial tissue probably does not occur. Wilrout and Satter (1972) have calculated that 62% of the glucose requirements of a lactating cow can be met from propionate. It is clear therefore that propionate conversion into glucose is a very important biochemical pathway in ruminants.

The ability of liver tissue to metabolize propionate increases as lactation progresses (Mathias and Elliot, 1967). Moreover, preliminary observations of Ricks et al., (1978) have shown that mitochondrial propionate activation in liver also increases as lactation progresses. This is probably a function of increased feed intake suggesting that the ability to utilize propionate for glucose synthesis might be controlled by suitable dietary manipulations; a fact of some significance to the dairy industry.

Amino acids can contribute from 13-30% of the glucose in sheep (Ford and Reilly. 1970; Reilly and Ford, 1971; Wolf and Bergman, 1972a, 1972b, 1972c). Similar values have been obtained for lactating cows by Hunter and Millison (1964). Black et al. (1968) and Egan and Black (1968) found that in lactating cows and goats 30-50% of the glucose turnover could arise from amino acids of which alanine and glutamine contributed 6-8% each. By potentiating propionate conversion to glucose, amino acids could be spared for more essential functions such as synthesis of milk protein.

Volatile fatty acids as physiological regulators of insulin and glucagon release - Insulin and glucagon are important regulatory hormones of liver carbohydrate metabolism in

non-ruminant animals. Insulin release is related to feeding in ruminants (Kamalu, 1970; Trenkle, 1970; Bassett et al., 1971; McAtee and Trenkle, 1971; Trenkle, 1972; Hove and Blom, 1973; Bassett, 1974a, 1974b; Trenkle, 1978). In view of the low levels of glucose absorbed from the gut, glucose is probably not a major physiological regulator of insulin release in ruminants (Bassett, 1975). The volatile fatty acids propionate and butyrate, but not acetate have been implicated as physiological regulators of insulin release in ruminants(Manns and Boda, 1967; Horino and Machlin, 1968; Trenkle, 1970; McAtee and Trenkle, 1971; Kamalu, 1975; Carstairs, 1978). Stimulation of insulin release by the volatile fatty acids does not occur in non-ruminants (Horino and Machlin, 1968). There is however, still considerable controversy as to the physiological importance of volatile fatty acids in insulin release. Stern et al. (1970) found that physiological quantities of volatile fatty acids administered via the gastro-intestinal tract were ineffective in eliciting insulin release. Bassett (1975) has pointed out that the insulinogenic actions of volatile fatty acids have only been observed, after injection or infusion of unphysiologically high levels. Trenkle (1978) however, has obtained an insulinogenic response to physiological concentrations of propionate and butyrate when these were infused intraruminally in fasted sheep. He has postulated that the lack of response obtained by Stern et al. (1970) was due to the ad libitum feeding practiced by

these investigators. Plasma insulin was therefore, already at high levels, and any additonal response to volatile fatty acids would be masked.

The physiological regulator of glucagon release in ruminants is unknown although it is known that plasma glucagon concentrations are related to feeding (Kamalu, 1970; Bassett, 1972). Lack of information on glucagon is due to the difficulty in measuring this hormone by radioimmuno-assay; both pancreatic and gut glucagon cross react in the assay. Bassett (1975) has postulated that since glucagon is a regulator of gluconeogenesis in the non-ruminant and since this gluconeogenesis is such an important process to the ruminant, this hormone may play an important role in maintaining hepatic output of glucose in ruminants.

In the non-ruminant, many of the effects of insulin and glucagon can be directly related to the activity of rate controlling enzymes of gluconeogenesis, glycolysis and glycogen metabolism. No data is available on the precise hormonal controls of these enzymes in ruminants. The only data available pertains to the levels of these enzymes under different physiological and nutritional states. A brief summary of these effects will be discussed here, in view of the importance of these enzymes in controlling the flux through the pathways of carbohydrate metabolism (Figure 3).

Phosphoenolpyruvate carboxykinase, a highly adaptive enzyme in non-ruminants, generally shows little adaptation in cattle (Ballard et al., 1969) or in sheep (Filsell et

al., 1969). However,Butler and Elliot (1970) did find that decreased feed intake was associated with lower levels of phosphoenolpyruvate carboxykinase in dairy cows. This enzyme also exhibits a very characteristic intracellular distribution dependent on the species. In rats 90% is found in the cytosol and 10% within the mitochondria (Ballard and Hanson, 1967); in ruminants the distribution is approximately equally divided between the two compartments (Ballard et al., 1969). These differences in intracellular distribution must have profound effects on the control of gluconeogenesis although these are not understood at present.

Adaptations of mitochondrial and cytosolic pyruvate carboxylase do occur in ruminants. In cattle enzyme activity increases with lactation and increases even further if the lactating animals are starved (Ballard et al., 1969). In sheep pyruvate carboxylase activity also increases with fasting (Filsell et al., 1969). Fatty acid coenzyme derivatives, particularly propionyl and butyrl CoA are produced in large quantities in ruminant liver by the action of acyl CoA synthetases (Cook et al., 1969; Ballard et al., 1969) and could potentially lead to an increase in pyruvate carboxylase activity under certain conditions, such as after feeding.

The activities of glucose-6-phosphatase and fructose-1-6-diphosphatase have been shown to increase in the liver and kidney of fasted sheep (Filsell et al., 1969).

Mackie and Campbell (1972) have found that glucose-6-phosphatase increases in lactating ewes. As already discussed propionate activation in ruminant liver does increase as lactation progresses (Ricks et al., 1978).

Despite these studies, which suggest that changes in gluconeogenic enzymes do occur in ruminants, albeit of smaller magnitude to those observed in the rat, Young et al. (1969), based on their studies in cattle, have interpreted their data to mean that there is little need for changes in gluconeogenic enzymes in ruminants because of the continuous requirement for gluconeogenesis in these animals.

Two isozymes are responsible for the phosphorylation and trapping of glucose as glucose-6-phosphate in the liver cell of the monogastric animal. These are hexokinase, a low K_m high affinity form (present in peripheral tissues also) and glucokinase, a high K_m low affinity form found only in liver tissue. Glucokinase is a highly adaptable enzyme whose activity is dependent on insulin. Diabetic animals have low glucokinase levels in liver tissue (Ballard et al., 1969). Glucokinase is the enzyme responsible for handling large influxes of glucose from the gut such as occurs after a meal (Ballard et al., 1969). Ruminant animals lack glucokinase; an adaptation to low quantities of glucose being absorbed from the gut (Ballard et al., 1969). Fetuses of all animals so far investigated, namely the rat, sheep and guinea pig also lack hepatic

glucokinase. This is probably because the fetuses of all species are supplied with a relatively constant supply of glucose from the mother and so have no need for such an enzyme (Ballard et al., 1969).

Pyruvate kinase is an important regulatory enzyme of glycolysis in non-ruminants. It is important that pyruvate kinase be under chronic inhibition when glucose synthesis occurs, otherwise all the phosphoenolpyruvate formed by the action of phosphenolpyruvate carboxykinase will be converted back to pyruvate. Pyruvate kinase activity would be expected to be low in ruminant liver.

Glycogen stored in the non-ruminant liver is a readily available source of glucose for use when dietary glucose levels are inadequate. Ballard et al., (1969) have found that during the development of the mature ruminant the activities of glycogen synthetase and phosphorylase fall, whereas this decrease does not occur in monogastric species such as the rat (Ballard and Oliver, 1963). Glycogen levels in the livers of lactating cows are extremely low relative to the amounts stored in non-ruminant liver; an observation which has led Ballard et al. (1969) to conclude that animals which do not have large fluctuations in blood glucose in response to feeding do not have the potential to store large amounts of glucose as glycogen.

Confusion exists in the literature as to how feeding, hormonal controls and carbohydrate metabolism are integrated in the ruminant. It is proposed that

immediately post-feeding insulin is released in response to propionate, butyrate, and amino acids being absorbed from the gastro-intestinal tract. This stimulates the peripheral uptake of acetate, glucose, and amino acids. Falling glucose levels perhaps stimulate glucagon secretion by the pancreas and so 2-4 hours after feeding gluconeogenesis is stimulated as has been observed (Bergman, 1973). This scheme would be compatible with the known effects of insulin and glucagon on the rate controlling enzymes of carbohydrate metabolism.

Lipogenesis - Ruminant animals are characterized by their inability to synthesize significant quantities of lipid in liver tissue (Ingle et al., 1972a, 1972b). Acetate, a primary substrate for lipogenesis in ruminants, is not activated by ruminant liver tissue (Quraishi and Cook, 1972).

The biochemical pathways of carbohydrate and lipid metabolism in ruminants and non-ruminants are shown in Figure 3.

Kidney metabolism

In ruminant animals, the kidney tissue does have the capacity to spare the utilization of glucose for energy generation since the trapping of acetate can occur in this tissue (Table 1).

It has been estimated that 8-10% of the total glucose synthesized by a ruminant animal can be made in the kidneys (Krebs and Yoshida, 1963; Kaufman and Bergman, 1968; Weidemann and Krebs.1969; Bergman, 1973; Bergman et

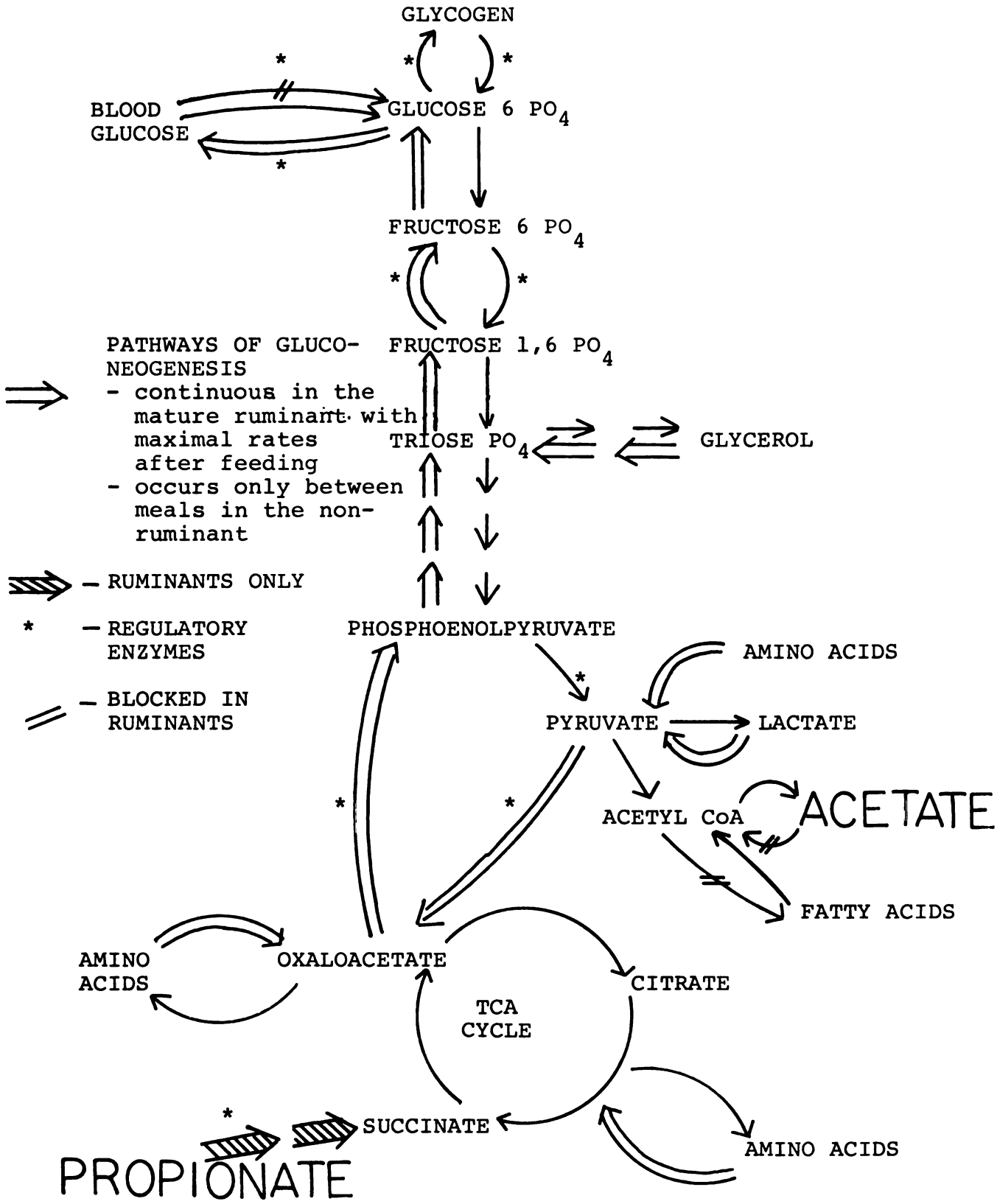


Figure 3 Biochemical pathways of carbohydrate and lipid metabolism operating in the liver of ruminant and non-ruminant animals.

al., 1973). This quantity may increase to 15% on fasting (Bergman, 1973). Renal gluconeogenesis in humans can increase even further during prolonged fasting (Owen et al., 1969). This suggests that under conditions of metabolic stress such as might occur in a high producing cow at peak lactation the kidney might synthesize considerable quantities of glucose. It is not likely, however, that propionate produced in the rumen is a substrate for renal gluconeogenesis. Lactate, pyruvate, glutamine, and glycerol have been shown to be important substrates for renal gluconeogenesis (Goodman et al., 1966; Kaufman, 1972).

Mammary gland metabolism

The major difference between ruminant and non-ruminant mammary tissue metabolism is that in the latter acetate can spare the action of glucose by furnishing energy as ATP via oxidation in the TCA cycle, and carbon skeletons for fatty acid synthesis as shown in Figure 4. In contrast, non-ruminant mammary tissue uses glucose as the major metabolic substrate for energy generation and both glucose and acetate for fatty acid synthesis (Bauman and Davis, 1974).

In the lactating state ruminant mammary tissue takes up tremendous quantities of acetate and glucose from the blood. Ruminant metabolism of these substrates is shown in Figure 4. Uptake of glucose is essential since a shortage of this metabolite leads to a marked reduction in

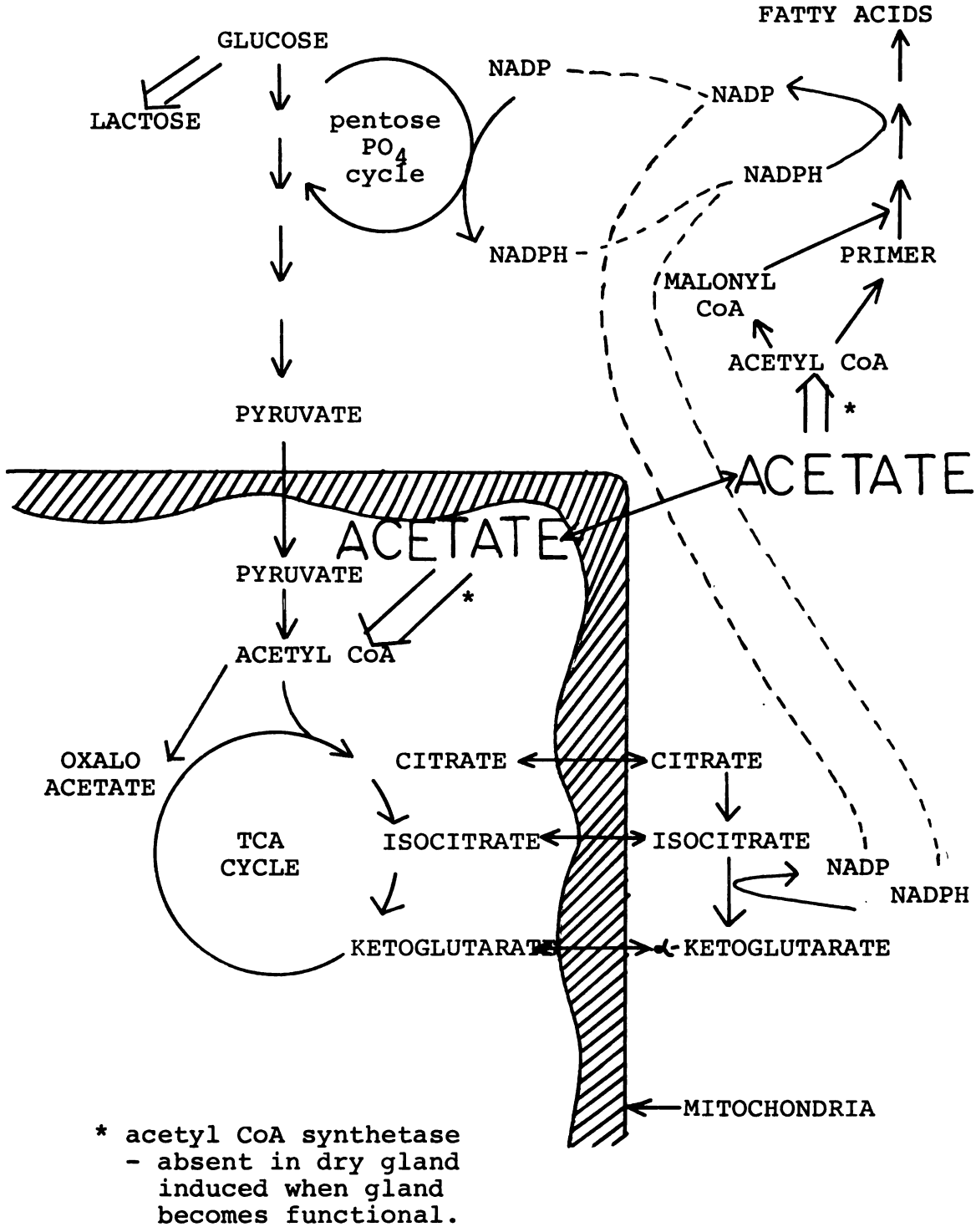


Figure 4 Biochemical pathways of carbohydrate and lipid metabolism in lactating ruminant mammary tissue.

volume of milk secreted (Hardwick et al., 1961, 1963; Linzell, 1967; Davis and Bauman, 1974). Annison and Linzell (1964) have estimated that 60-85% of the total glucose available to the animal will be taken up by the lactating goat mammary gland. This uptake will occur independent of insulin status (Hove, 1978). Approximately 50-60% of this will be used for lactose synthesis, 23-30% will be metabolized via the pentose phosphate pathway, and less than 10% via the glycolytic pathway (Wood et al., 1965; Linzell, 1968). This is in marked contrast to values obtained with non-ruminant species where although about the same amount of glucose is used for lactose synthesis approximately equal amounts of glucose are metabolized via the glycolytic and pentose phosphate pathways (Abraham and Chaikoff, 1959; McLean, 1964; Davis and Bauman, 1974). Smith (1971) and Chesworth and Smith (1971) have pointed out that probably little glucose is oxidized to carbon dioxide via the TCA cycle in ruminant mammary tissue and glucose carbon that does enter the TCA cycle does so as oxaloacetate rather than acetyl CoA. There is no evidence that gluconeogenesis occurs in ruminant mammary tissue (Scott et al., 1976).

Approximately 41% of the total acetate available to the body is extracted by the lactating goat udder (Davis and Bauman, 1974). The enzyme responsible for trapping this acetate is acetyl CoA synthetase (Qureshi and Cook, 1975). The enzyme occurs in both the mitochondrial and cytosolic

fractions of cow and goat mammary tissue (Marinez et al., 1976; Ricks et al., 1978). Enzyme activity is negligible in the dry gland, increases just prior to parturition, and then follows the lactational curve. Enzyme activity can be reinstated by hormone treatment (Marinez et al., 1976).

The role that acetate plays in mammary tissue metabolism depends on whether the acetate is trapped as acetyl CoA in the cytosol or mitochondrion (Figure 2).

Mitochondrial acetate will generate energy in the form of ATP via the TCA cycle and reducing equivalents for fatty acid synthesis in the cytosol. In lactating goats acetate oxidation can account for 23-27% of the total mammary carbon dioxide (Annison and Linzell, 1964; Annison et al., 1967), and glucose oxidation for 29-49%.

Cytosolic activation of acetate yields a major source of carbon for de novo synthesis of fatty acids in ruminant mammary tissue (Folley and French, 1950; Popjak et al., 1951a, 1951b). From 35-45% of the total milk fatty acids can be synthesized from acetate (Hardwick et al., 1963; Annison and Linzell, 1964; Palmquist et al., 1969; Davis and Bauman, 1974). Numerous studies have shown that glucose cannot serve as a carbon source for fatty acid synthesis in ruminant mammary tissue (Hardwick et al., 1963; Bauman et al., 1970). Most of the remaining fatty acids found in milk fat are taken up preformed from the blood. The biosynthesis of milk fat has been reviewed by Bauman and Davis (1974).

Adipose tissue metabolism

In ruminant animals the major site of fatty acid synthesis is adipose tissue (Ingle et al., 1972a, 1972b) whereas in chickens the major site of fatty acid synthesis is the liver (Allen et al.,1976) and in rats fatty acid synthesis occurs about equally in liver and adipose tissue (Leveille, 1967). This shift of fatty acid synthesis to adipose tissue in ruminants allows ruminants to maximize the potential for gluconeogenesis in liver (Ballard et al., 1969; Ingle et al., 1972a; Bauman, 1976). Rates of fatty acid synthesis do vary between the different adipose depots (Ingle et al., 1972b). Rates are highest in the young growing animal (Allen et al.,1976).

It is well established that in the non-ruminant glucose is the primary carbon source for fatty acid synthesis. In addition the reducing equivalents required for lipid synthesis are generated via the pentose phosphate shunt and the malate transhydrogenation cycle from glucose. These pathways are shown in Figure 5. In ruminants glucose has been excluded both as the primary carbon source for fatty acid synthesis (Hanson and Ballard, 1968; Ingle et al., 1972b; Hood et al., 1972) and as a primary source of reducing equivalents (Bauman, 1976).

Ruminant tissues that actively synthesize lipid such as adipose or lactating mammary tissue contain negligible levels of ATP citrate-lyase and NADP-malate dehydrogenase.

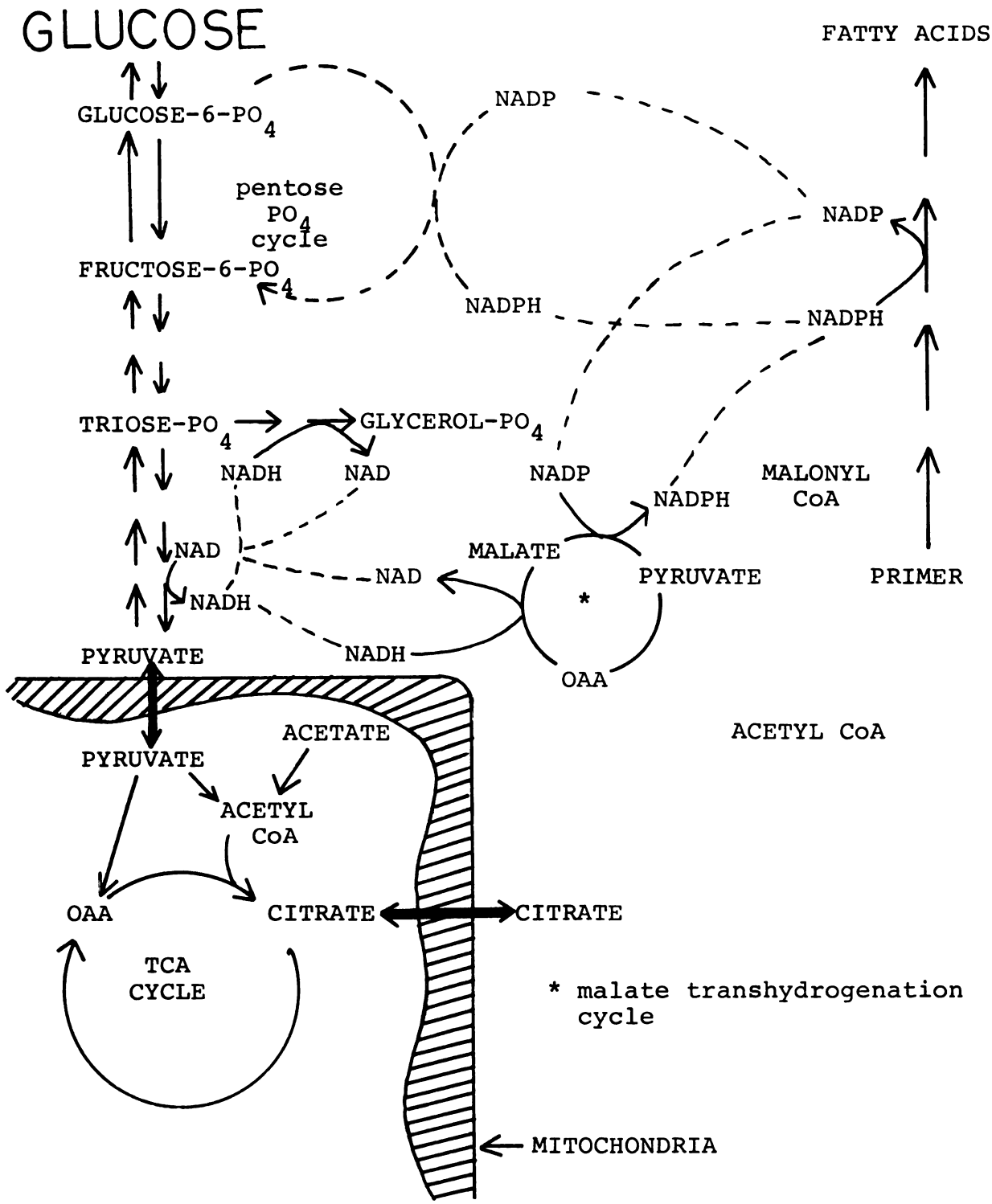


Figure 5 Biochemical pathways of fatty acid synthesis in non-ruminant liver tissue

The former enzyme is required for the shuttling of glucose carbon across the mitochondrion to the cytosol; the site of fatty acid synthesis. The latter enzyme is involved in the transfer of reducing equivalents. Thus, in ruminants glucose is excluded as a carbon source for fatty acid synthesis. Acetate in the cytosol is used as the primary substrate for fatty acid synthesis (Hanson and Ballard, 1967). The rate limiting enzyme for fatty acid synthesis in both ruminants and non-ruminants is acetyl CoA carboxylase (Ingle et al., 1973; Bauman and Davis, 1974). Acetyl CoA synthetase may be regulatory in some instances (Howard et al., 1974).

Ingle et al. (1972b) suggested that the reducing equivalents required for fatty acid synthesis in ruminant tissues are generated via the action of isocitrate dehydrogenase. Mitochondrial acetate serves as the substrate for the supply of reducing equivalents in ruminants.

Substantial quantities of acetate can be oxidized to carbon dioxide in bovine adipose tissue (Hood et al., 1972) and therefore acetate can supply energy in the form of ATP for adipose metabolism.

The pathways for fatty acid synthesis and reducing equivalent generation from acetate in ruminant tissue are shown in Figure 6.

The ruminant animal by using acetate as a source of reducing equivalents, as a source of carbon for fatty acid synthesis, and as a source of energy, has conserved glucose for more essential functions. The intermediary

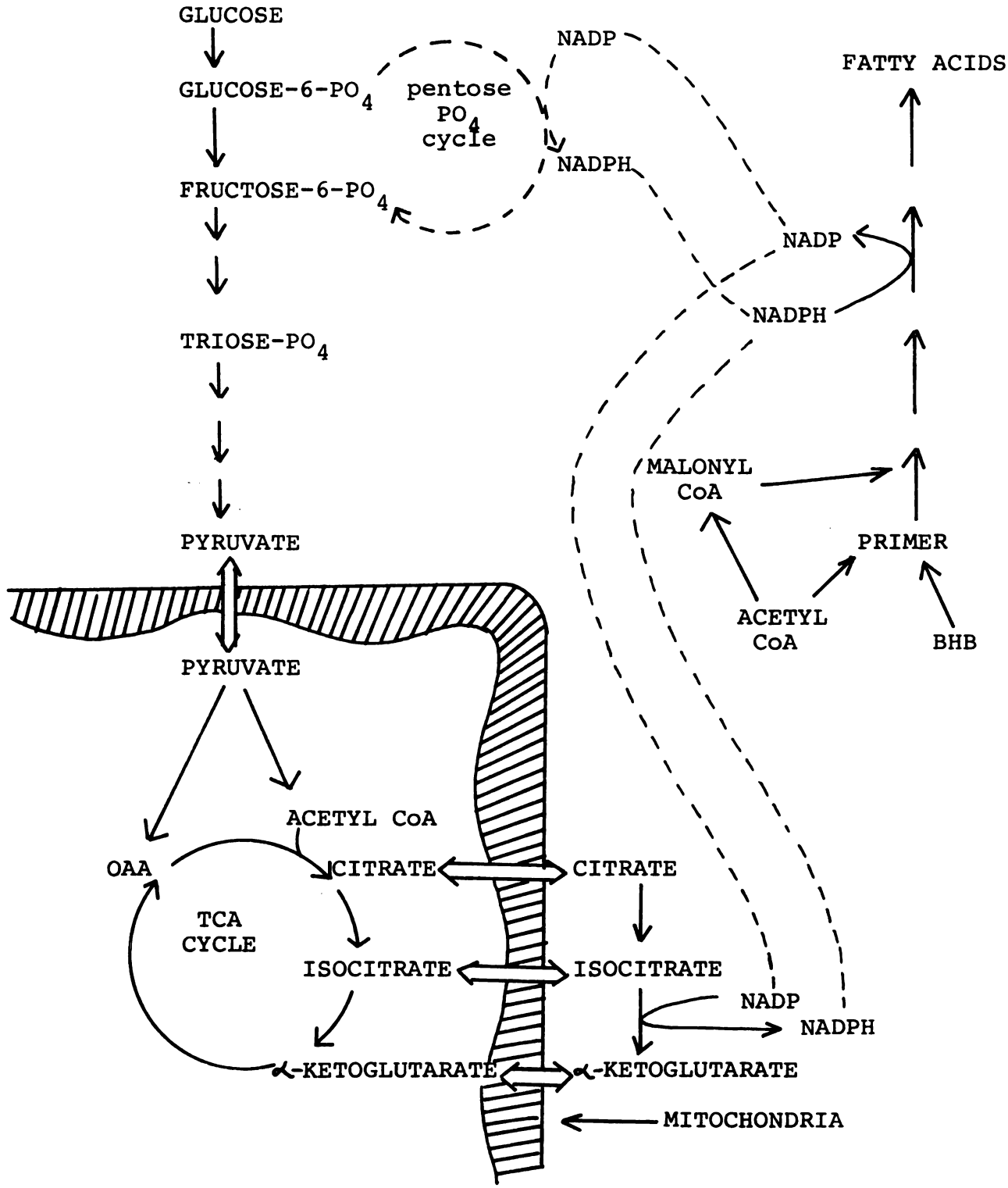


Figure 6 Biochemical pathways of fatty acid synthesis in ruminant adipose tissue

metabolism of adipose tissue in meat producing animals has been reviewed by Allen et al., (1976) and Bauman (1976).

Metabolism in other tissues

Extra-hepatic ruminant tissues obtain a large portion of their energy by the aerobic oxidation of acetate (Warner, 1964). For example, cardiac muscle which uses primarily long chain fatty acids as energy furnishing substrates has an active mitochondrial acetyl CoA synthetase (Campagnari and Webster, 1963) and thus can utilize large quantities of acetate for the synthesis of ATP. As already described both kidney and mammary tissue can oxidize acetate for energy.

Some ruminant tissues probably do not oxidize acetate. Since the erythrocyte lacks mitochondria it cannot use acetate as a source of energy but must depend on the anaerobic metabolism of glucose for energy. Skeletal muscle probably does not utilize acetate either (Cook et al., 1969).

The brain is a unique case since in non-ruminants it has been shown to be absolutely dependent on glucose (Owen et al., 1967). In ruminants acetate probably does not spare the action of glucose in brain tissue at the level of the TCA cycle even though it should be freely permeable to the blood brain barrier (McClymont and Setchell, 1956; Setchell, 1961). This is because mitochondrial acetyl CoA hydrolase levels in brain tissue are high relative to the synthetase levels (Quraishi and Cook, 1972). Owen et

al., (1967) have shown that in humans under prolonged fasting conditions the brain can adapt to using ketone bodies as a primary source of energy. Since ruminants are characterized by low blood glucose levels this might suggest that ruminant brain tissue uses ketone bodies under normal feeding conditions. Brain tissue contains a high level of lipid material. It is not known whether acetate can spare glucose action in ruminants by incorporation into brain lipids via cytosolic activation.

Metabolism in the Young Calf

It is generally agreed that the newborn ruminant has a system of metabolism similar to the monogastric animal and that the shifts in the patterns of intermediary metabolism occur as the microbial fermentation process becomes established.

Volatile fatty acid utilization

The newborn ruminant has an undeveloped rumen and little volatile fatty acid production. Concentrations of total volatile fatty acids produced by rumen fermentation increase with age reaching a maximum in the calf about a week after weaning (McCarthy and Kesler, 1956). Volatile fatty acid levels in peripheral blood also increase with age although these are somewhat more variable (McCarthy and Kesler, 1956).

There is some evidence that the pre-ruminant calf can utilize volatile fatty acids (Liang et al, 1967) which

are probably produced in the large intestine (Huber and Moore, 1964). Calves maintained on an all milk diet for up to 80 days can derive 20-30% of their energy from volatile fatty acids (Young et al., 1965). Little information is available as to whether the acyl CoA synthetases are constitutive i.e. present at birth or whether these enzymes increase as the fermentation process becomes established. Warshaw (1970) has demonstrated that bovine fetal heart tissue is deficient in its ability to activate acetate since acetyl CoA synthetase levels are low.

Glucose homeostasis

In young calves blood glucose levels fall from a value of 100 mg/ml at birth to about 60 mg/ml at 6 weeks of age (McCandles and Dye, 1950; McCarthy and Kesler, 1956). This has been associated with a change in energy metabolism of the young calf, whereby volatile fatty acids replace glucose as the major energy furnishing substrates. Glucose homeostasis in the pre-ruminant calf is similar to that of an animal possessing a monogastric type of metabolism (Dollar and Porter, 19571 Ballard et al., 1969).

Early workers attributed the decrease in glycemia to the growth of the rumen. However, milk fed calves (Jacobson et al., 1951) and calves fed their solid food by abomasal fistula (Nicolai and Stewart, 1965) showed blood sugar levels similar to calves raised on high forage diets. This suggested that neither rumen development nor the

absorption of volatile fatty acids into the circulation influenced the development of hypoglycemia in the young calf. Liang et al., (1967) have critized these conclusions on the basis that the abomasal feeding practiced in the latter case would have resulted in intestinal fermentation and that this may also have occurred in the calves in the former case before they were put on the experiment.

The fall in blood glucose has been attributed to changes in both the glucose content of the red cells and a slower decline in plasma levels (Reid, 1953). The decrease in the former has been attributed to the replacement of fetal cells by an adult type (Tucker, 1963) and in the latter to a progressive decrease in glucose entry rate (Ballard et al., 1969).

Hepatic glucokinase, the enzyme responsible for trapping large quantities of glucose from the gut, is absent in all species tested at birth (Ballard et al., 1969). This has been attributed to the fact that a constant level of glucose is provided to the fetus from the maternal circulation. After birth hepatic glucokinase begins to increase in the non-ruminant but fails to develop in the ruminant (Ballard et al., 1969). Since addition of glucose to the diet of a suckling animal fails to induce the enzyme it has been postulated that glucose concentration per se is not the only factor involved in the development of glucokinase activity (Walker, 1965).

Lipogenesis

Both the fetal and newborn ruminant have relatively high rates of lipogenesis in liver (Hanson and Ballard, 1967, 1968) and have the ability to utilize glucose for lipid synthesis (Ballard et al., 1969). ATP citrate lyase and NADP malate dehydrogenase are present in liver tissue in the young calf (Ballard et al., 1969).

The shift in metabolism to the adult form of metabolism occurs at the time of weaning when rumen development occurs (Muramatu et al., 1970).

MATERIALS AND METHODS

Reagents

Acetyl CoA, ATP, Tris (Trizma base), AMP, bovine serum albumin, valeric acid,fumaric acid, 2-mercaptoethanol and all reagents for polyacrylamide electrophoresis were purchased from Sigma Chemical Co., St. Louis, Mo. 5'-AMP-Sepharose 4B and ovalbumin were obtained from Pharmacia Fine Chemicals Inc., 800 Centennial Av., Piscataway, NJ. Potassium acetate and ammonium sulfate were purchased from Mallinckrodt, St Louis, Mo. Potassium propionate was purchased from ICN Pharmaceuticals Inc., Plainview, NY. Hexanoic acid, heptanoic acid, octanoic acid, acyrlic acid, maleic and crotonic acids were purchased from Eastman Organic Chemicals, Rochester 3, NY. and benzoic acid from J. T. Baker Co. Phillipsburg NJ. Dialsis tubing was obtained from Union Carbide Corporation, 6733 West 65th St., Chicago, ILL. DEAE-23 cellulose and cellulose phosphate P11 (phosphocellulose) were obtained from Whatman Biochemicals Ltd, Springfield Mill, Maidstone, Kent, England. Alkyl agaroses (agarose-C_n series) for hydrophobic chromatography were obtained in kit form from Miles Laboratories, Elkhart, IND.

Experimental Design

For the purification studies tissue from lactating Holstein cows was obtained from a local abattoir. No data was available on the previous history of these animals.

For the second experiment, involving the measurement of volatile fatty acid activating enzymes in the young animal, Hostein bull calves were used. These calves were procured from the Michigan State University dairy farm or from Dr. T. Spike. Calves were fed colostrum after birth and weaned as early as possible by offering ad libitum, alfalfa hay and corn starter at one week of age. Calves were bedded on straw. Groups of 3-5 calves were slaughtered at -14,0,1,7,14,40,60 and 120 days of age. Animals were not allowed access to feed prior to slaughter. An additional group of calves were maintained on whole milk fed twice daily at a rate of 4 Kg/100 Kg body weight per day. Five calves were slaughtered at 60 days of age and eight calves at 120 days of age. At slaughter jugular blood was collected in heparinized tubes (Becton Dickenson Inc., Rutherford, NJ).

Enzyme Assay

Enzyme activity was determined using the method of Mahler et al.(1953). In this reaction the disappearance of the free -SH group of coenzyme A is measured using the nitroprusside reagent prepared according to the method of Grunert and Phillips (1951). The complete reaction mixture

contained 2.5 μmoles $MgCl_2$, 1.1 μmoles ATP, 0.17 μmoles coenzyme A, 7.5 μmoles Tris (hydroxy-methyl) amino methane hydrochloride buffer and 5.0 μmoles of substrate, either potassium acetate, propionate, butyrate or valerate in a total volume of 0.15 ml. When either kidney or liver tissues were being assayed the ATP concentration was increased to 1.165 μmoles. Blank tubes did not contain substrate. Standard tubes did not contain coenzyme A. From 50 to 4 μg of enzyme protein were used.

The reaction was carried out at 37° and initiated by the addition of enzyme to tubes that had been preincubated for 1 minute. After incubation for 10 minutes the reaction was terminated by the addition of 2.8 ml of nitroprusside reagent. The optical density was measured precisely 30 seconds after reaction termination at 520 mμ using a Coleman Junior Spectrophotometer. The difference in optical density between the standard and the complete reaction mixture is the measure of enzyme activity. An optical density of 0.185 corresponds to the disappearance of 0.01 μmoles of coenzyme A (Qureshi, 1971). Enzyme concentration was adjusted to give an optical density of from 0.075 to 0.250. Withing this range OD_{520} is proportional to enzyme concentration. One unit of enzyme is defined as the amount which catalyzes the disappearance of 1 nmole of coenzyme A per minute. The OD_{520} was converted to units by multiplying by a factor of 3.243. The specific activity represents units of enzyme per mg of protein.

Protein Determinations

Protein was determined by the method of Lowry et al. (1951) using a Coleman Junior Spectrophotometer. During column chromatography the protein content of the effluent was measured in the various fractions using the method of Warburg and Christian (1941). A Bechman DB-G grating spectrophotometer was used.

Isolation of Mitochondria

After removal from the animal the tissue was transproted on ice for preparation in the laboratory. All subsequent steps were carried out at 4^{o}.

For the purification studies the tissues were sliced and then ground using a meat grinder. The ground tissue was then homogenized in a Waring blender using 1 part tissue to 2 parts of 0.13M KCl containing 2.5mM 2-mercaptoethanol and adjusted to pH 8.0 with 1 N ammonium hydroxide. Homogenization was carried out for 10 seconds on medium and 10 seconds on low. The homogenate was transferred to one liter bottles and centrifuged at 1000xg in an MSE centrifuge for 15 minutes. After centrifugation the 1000xg supernatant was filtered through 8 layers of cheesecloth and recentrifuged at 20,000xg in a Sorvall RC-2B centrifuge for 30 minutes. The 20,000xg pellet, composed of mitochondria, was resuspended in 0.13 M KCl containing 2.5 mM mercaptoethanol and 10% glycerol (pH 8.0). For each gram of mitochondrial pellet 3 ml of buffer was used and the

resuspension performed using a teflon homogeniser. Fatty acid activating enzymes were liberated from the mitochondria using a sonifier cell disrupter (Heat Systems Ultrasonic, Inc., L.I. NY). This process was carried out using the standard horn immersed in 300 ml quantities of mitochondrial suspension. Sonication time was for 2 minutes at a setting of 3. The sonicated extract was then centrifuged at 30,000xg for 30 minutes. The supernatant was designated the mitochondrial extract. In some cases the enzymes were liberated by freezing and thawing as described by Qureshi (1971).

For the calf experiment this procedure was modified so that the cytosolic fatty acid activating enzymes could be stabilized. Ten gram samples of heart, kidney cortex and liver were prepared by homogenizing in 20 ml of KCl pH 8.0 containing 2.5 mM 2-mercaptoehtanol and 10% glycerol. The samples were centrifuged at 2,000xg for 15 minutes, the supernatant filtered through 8 layers of cheesecloth and then recentrifuged at 30,000xg for 30 minutes. The resulting precipitate was resuspended in a known volume of the above buffer. This fraction was composed of mitochondria. The acyl CoA synthetases were liberated from the mitochondria by sonication for 30 seconds using a micro-tip (setting 3). The resulting sample was assayed using acetate, propionate, butyrate and valerate as substrates to monitor enzyme activity. Using the same substrates the 30,000xg supernatant (cytosol fraction) was also assayed for fatty

acid activating enzymes. This procedure ensured that the cytosolic forms of the enzymes were not denatured during sample preparation.

Ammonium Sulfate Fractionation

To the mitochondrial extract 243 g of solid ammonium sulfate was added slowly with stirring for each liter of solution. The pH was then adjusted to 8.0 with 1 N ammonium hydroxide and the solution allowed to stir gently for one hour. The solution was then centrifuged for 30 minutes at 30,000xg. To the supernatant a further 285 g of solid ammonium sulfate was added per liter of solution, allowed to stir gently for one hour, and then centrifuged as before. The pellet so obtained (80% precipitate) was resuspended in 0.05 M Tris-HCl containing 2.5 mM 2-mercaptoethanol and 10% glycerol and stored frozen at -20^{o} until required for chromatography.

Column Chromatographic Techniques

DEAE-23 cellulose chromatography

DEAE-23 cellulose was precycled, degassed and equilibrated as described in the Whatman information leaflet on advance ion exchange celluloses. Column dimensions were 2 cm x 40 cm. For purification of enzymes from liver tissue the column dimensions were 2.5 cm x 47 cm. Column equilibration was carried out overnight using 0.005 M Tris-HCl containing 2.5 mM 2-mercaptoethanol and 10% glycerol pH 7.5.

Ammonium sulfate precipitate was dialyzed against the equilibration buffer for 40 minutes and then diluted to 1.5-2.0 mg/ml. Approximately 400 mg of protein were used; for liver enzyme purification this was increased to 800 mg. The sample was added to the column, washed on with 140 ml of equilibration buffer followed by 160 ml of 0.01 M Tris-HCl containing 2.5 mM 2-mercaptoethanol and 10% glycerol and then eluted with 600 ml of a linear KCl gradient of 0-0.6 M in 2.5 mM 2-mercaptoethanol, 0.01 M Tris-HCl and 10% glycerol, pH 7.5 at a flow rate of 20 ml/hr. These volumes of buffers were increased proportionally when the column size was 2.5 cm x 47 cm. Fractions containing enzyme with the highest specific activity were combined and concentrated using ultrafiltration with a Dia-flo cell. The concentrated protein was stored at -20°.

Phosphocellulsoe chromatography

Phosphocellulose was pretreated as recommended by Burgess (1969) and fully equilibrated in 0.01 M potassium phosphate buffer pH 6.5 containing 10% glycerol and 2.5 mM 2-mercaptoethanol. Ammonium sulfate precipitate was dialyzed for 40 minutes against the equilibration buffer and then diluted to 10 mg/ml. The column dimensions were 1.2 cm x 20 cm and the flow rate 25 ml/hr. Approximately 100 mg of protein was applied to the column, washed on with 50 ml of 0.01 M potassium phosphate buffer containing 10% glycerol and 2.5 mM 2-mercaptoethanol, pH 6.5, and then

eluted with a linear KCl gradient of 0-2.0 M in the same buffer.

Hydrophobic chromatography

Six columns(1.4 cm x 8 cm) each containing 1 ml of a different alkyl agarose (Agarose-C_n) were used (n equal to 0,2,4,6,8,10). The columns were washed in 2 M urea (5 ml), water (25 ml) and then equilibrated in 1 M potassium phosphate buffer containing 10% glycerol and 2.5 mM 2-mercaptoethanol pH 7.5. Ammonium sulfate precipitate was dialyzed for 40 minutes against equilibration buffer and then applied directly to the top of each column without dilution. The enzyme was washed onto each column with 2.5 ml of equilibrating buffer and the complete 2.5 ml collected in one tube. Each column was eluted with 2.5 ml of 50 mM potassium phosphate buffer pH 7.5 containing 10% glycerol and 2.5 mM 2-mercaptoethanol and this 2.5 ml collected in one tube. Flow rate was 2 ml/hr.

Calcium phosphate gel chromatography

Calcium phosphate gel was prepared according to the method of Miller et al. (1965). Column dimensions were 3 cm x 15 cm. The column was equilibrated overnight in 0.001 M potassium phosphate buffer pH 7.0 containing 10% glycerol and 2.5 mM 2-mercaptoethanol. The extract from a DEAE-23 cellulose column was dialyzed for 40 minutes against equilibration buffer. The dialyzed sample was diluted to approximately 0.5-1.0 mg/ml and 50-150 mg of protein applied

to the column. The enzyme activity was eluted with a stepwise gradient of increasing concentrations of potassium phosphate buffer pH 7.0 from 0.001 M to 0.5 M. All buffers contained 10% glycerol and 2.5 mM 2-mercaptoethanol. Fractions with the highest specific activity were pooled and concentrated by ultrafiltration using the Dia-flo cell. The enzyme protein was stored frozen at -20° in 0.05 M Tris-HCl containing 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was approximately 100 ml/hr.

Affinity chromatography using 5'-AMP-Sepharose 4B

The gel was reconstituted by stirring 1 g of dry powder in 100 ml of 0.1 M potassium phosphate buffer pH 7.0 for 1 hr at 0°. The column dimensions were 0.6 cm x 20 cm. After packing at room temperature the column contained 3.0 ml of packed gel. The column was washed with several volumes of the above buffer. Column equilibration was carried out using 0.001 M potassium phosphate buffer pH 7.0 containing 10% glycerol and 2.5 mM 2-mercaptoethanol. The ammonium sulfate precipitate was dialyzed for 40 minutes against the equilibration buffer and then applied directly to the column without dilution. Approximately 25-30 mg of protein was used. Equilibration buffer was used to wash the protein onto the column. The column was eluted using either 0.1 M or 0.6 M KCl in 0.001 M potassium phosphate buffer pH 7.0 containing 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was

15 ml/hr.

Sucrose Density Centrifugation

The molecular weights of the various enzyme preparations were determined by the technique of sucrose density centrifugation as described by Martin and Ames (1961). Linear sucrose gradients of 5-20% sucrose in 0.05 M Tris-HCl pH 7.5 in a total volumne of 4.4 ml were prepared in cellulose nitrate tubes. Enzyme protein was applied in the same buffer to the top of the gradients. Centrifugation was carried out at 50,000 rpm (246,000xg) for 12 hours at 4^{o} in a Beckman SW-56 rotor. Ovalbumin and bovine serum albumin were used as markers. After centrifugation the bottum of each tube was punctured and eight drops per fraction collected. Protein content was measured using the method of Warburg and Christian (1941). For tubes containing enzyme all fractions were assayed for activity.

Electrophoresis

Enzyme puriity was assessed using the technique of polyacrylamide disc electrophoresis as outlined by Maurer (1971). The following solutions were used in gel preparation. Solution A contained 48.0 ml 1 N HCl, 36.6 g Tris, 0.23 ml TEMED and water to 100 ml. Solution B contained 28.0 g acrylamide, 0.735 g Bis and water to 100 ml. Solution C contained 0.14 g ammonium persulfate per 100 ml solution. Solution D contained 22.2 g acrylamide, 0.735 g Bis and water to 100 ml. For a 7% gel one part solution A,

two parts solution B, one part water and four parts of solution C were mixed. For a 5.5%gel one part solution A, two parts solution D, one part of water and four parts of C were mixed. The gel mixture was carefully poured in tubes (0.5 cm x 10 cm) to a length of 9 cm. If the gels did not polymerise within 30 minutes they were considered to be heterogeneous and discarded. The electrode buffer used contained 3 g Tris and 14.4 g glycine and water to one liter. Gel tubes were placed in the electrophoretic apparatus and covered with electrode buffer (1:10 aqueous dilution of stock electrode buffer was used). Enzyme proteing was made dense by the addition of a few crystals of sucrose and then layered carefully onto each gel. Bromophenol blue (0.05%) was used as the tracking dye. Electrophoresis was carried out at room temperature with a current of 3-4 ma/tube until the tracking dye had migrated approximately 8-9 cm. After electrophoresis gels were removed carefully from the gel tubes and the position of the tracking dye marked. The gels were then placed in a 12.5% solution of trichloroacetic acid (TCA) for 30 minutes, transferred to a staining solution of 1% aqueous stock solution of coomassie blue ditlued 1:20 with 12.5% TCA for 30 minutes to one hour and then stored in 10% TCA (Chrambach et al., 1967). On storage some bands have a tendency to fade. These gels could be restained as outlined above. In some cases the periodic acid - Shiff (PAS) staining technique of

Hotchkiss (1948) for the detection of carbohydrate components was used following electrophoresis on acrylamide gels. The procedure used has been described in detail by Stamoudis (1973).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was used to determine sub-unit molecular weight of purified enzyme protein (Welton and Felgner, 1975). Gels were prepared according to the procedure of Fairbanks et al. (1971). The following solutions were used. Solution A contained 40 g of acrylamide, 1.5 g Bis and water to 100 ml. Solution B contained Tris (0.4 M), sodium acetate (0.2 M), and EDTA (0.02 M), pH was adjusted to 7.4 with acetic acid. Solution C contained 100 g SDS per liter of water. Solution D contained 1.5% ammonium persulfate. Solution E contained 0.5% TEMED. Gels were prepared by mixing solution A (5.6 ml), solution B (4.0 ml), solution C (4.0 ml), water (20.4 ml), soution D (4.0 ml) and solution E (2.0 ml). Solutions D and E were always added last. Gel tubes were filled to length of 9 cm and then layered with 50 µl of a freshly prepared solution containing 0.1% SDS, 0.15% ammonium persulfate and 0.05% TEMED. Polymerization usually occurred within 45 minutes. At this time the overlay was poured off and replaced with electrophoresis buffer. Electrophoresis buffer was prepared by mixing 100 ml of solution B with 100 ml of solution C and 800 ml of water. Gels were allowed to sit overnight before use and could be stored for at least 5 days. All

gels were pre-electrophoresed for 30 minutes at 3-4 ma per gel prior to application of the sample. Samples were prepared by dialysis against 10 mM Tris, 1 mM EDTA pH 7.5. Samples and standards were then made 1% SDS, 5% sucrose, 10 mM Tris, 1 mM EDTA and 2% 2-mercaptoethanol and heated in a boiling water bath for 15 minutes. Protein concentration within the sample mix was 2-5 mg/ml. Pyronin B was used as the tracking dye. For a 1 ml volume of sample or standard 0.02 ml of a 0.05% pyronin B in 5% sucrose was added. From 25 to 75 μg protein was applied to the gel in volumes not exceeding 0.1 ml. Electrophoresis was carried out at room temperature at 3-4 ma/gel for 3-4 hours or until the tracking dye had migrated 8 cm. After electrophoresis the gels were fixed in 10% TCA overnight and then allowed to stand for 6 hours in 10 ml of 15% TCA and 5 ml of 1.2% coomassie blue in methanol. The gels were destained in a solution of 10% TCA in 33% methanol and then stored in 10% TCA. A plot of log molecular weight versus relative migration is linear for proteins of molecular weight ranging from 20,000-130,000.

Gas Liquid Chromatography

A Hewlett Packard Model No. 5730A gas liquid chromatograph (Hewlett Packard, Avondale, PA) equipped with a flame ionization detector was used for all analyses. Peak area was measured by an electronic integrator. Unknowns were calculated by comparing peak areas of standard mixtures with areas of unknowns.

Monosaccharide components of purified enzyme proteins

Purified acetyl CoA synthetase prepared from bovine heart mitochondria was quantified for the presence of carbohydrate residues by gas chromatographic analysis of the trimethylsilyl derivatives. The procedure used has been described in detail by Stamoudis (1973). From 2 to 5 mg of purified enzyme protein was used. The column used was a 2.7 m x 1.8 mm i.d glass column packed with chromasorb W containing 3% OV-1 (Applied Science Lab. Inc. P.O. Box 440, State College, PA). Nitrogen at a flow rate of 30 ml/minute was used as the carrier gas. Isothermal chromatography at 160^{o} and 190^{0} was employed.

Plasma acetate

Plasma acetate was measured using a modification of the ethanolic extraction procedure of Remesy and Demigne (1973). Ten ml of plasma was mixed thoroughly with 50 ml ethanol. The resulting solution was centrifuged for 15 minutes at 1,000xg. The supernatant was transferred to a round bottomed flask and made alkaline by the addition of 100 µl of 2 M sodium hydroxide. The sample was then evaporated under vacuum at 20^{o}. The dry residue was redissolved in 830 µl of water. Just prior to injection onto the column 166 µl of 25% ortho-phosphoric acid was added to each flask. This minimized the risk of volatilization of acetate in the sample. The final preparation thus contained 10X the concentration of acetate than was contained in the original sample. From 1-2 µl of sample was injected onto the

column. Standard solutions of acetate were prepared with approximately the same quantity of acetate as was found in calf plasma. They were subjected to the same treatment as the plasma samples. 2-methyl butyric acid was used as the internal standard.

A glass column 2.7 m long, 2 mm i.d packed with 3% carbowax 20M, 0.5% H_3PO_4 on 60/80 mesh carbopack B (Supelco Inc., Bellefonte, PA) was used. Isothermal chromatography at 170^{o} was employed with nitrogen carrier gas flow rate of 25 ml/min.

Thiobarbituric Acid Assay

The method of Warren (1959) was employed to determine the sialic acid content of a purified protein. Sialic acid was released from the protein by acid hydrolysis using 0.1 N H_2SO_4 at 80^{o} for 1 hour. Samples and standards contained from 2-18 µg of sialic acid in a total volume of 0.2 ml. To each sample 0.1 ml of a 0.2 M sodium metaperiodate in 9 M phosphoric acid was added, the tubes vortexed and allowed to stand at room temperature for 20 minutes. One ml of a 10% solution of sodium arsenite in a solution of 0.5 M sodium sulfate and 0.1 N H_2SO_4 was then added and the tubes shaken until the brown color dispersed. Three ml of 0.6% thiobarbituric acid in 0.5 M sodium sulfate was then added, the tubes shaken, capped with glass bulbs and then heated in a boiling water bath for 15 minutes. After cooling for 5 minutes in a water bath at room temperature

the contents of each tube were extracted with 4.3 ml of cyclohexanone. The aqueous and organic phases were separated by centrifugation and the upper organic phases transferred to cuvettes and the optical density determined at 549 mμ using a Coleman Junior Spectrophotometer. Color intensity is linear in the range 0.01 - 0.06 μmole of sialic acid.

Statistical Analyses

Assay variability

An optical density of 0.037 was significant ($P < .05$).

Enzyme kinetic data

Michaelis constants (K_m) and maximal velocities (V_{max}) were determined from Lineweaver-Burk and Eadie-Scatchard plots using linear regression. Theoretical curves were obtained by using the K_m and V_{max} values derived from the Eadie-Scatchard plot.

Effect of age and diet on volatile fatty acid activating enzymes in the young calf.

Tests for the significance of the effects of age on the volatile fatty acid activating enzymes were determined using Tukey's test for comparison of all means. The effect of diet was analyzed by a least squares analysis of variance. Correlation coefficients were determined to measure the relationship between blood acetate concentration(mM) and enzyme activity.

RESULTS

Purification and Characterization of the Fatty Acid Activating Enzymes of Bovine Heart Mitochondria

Initial studies were directed towards establishing if the enzyme from heart mitochondria which exhibits a pattern of substrate specificity similar to the mammary gland mitochondrial enzyme could be purified by the established procedures of Qureshi and Cook (1975) and whether the enzyme was a glycoprotein as established for the mammary gland enzyme (Stamoudis and Cook, 1975).

The purification procedures used did not utilize glycerol in the buffers. Enzyme activity was liberated by freezing and thawing the mitochondrial suspension three times (Table 3) over a period of one month. After fractionation with ammonium sulfate (Table 3) the enzyme was purified by column chromatography using DEAE-23 cellulose and enzyme activity in column effluents was monitored using acetate as the substrate. Enzyme activity was associated with one peak. Tubes containing enzyme activity were pooled and concentrated by ultrafiltration. The concentrated enzyme activated both acetate and propionate but exhibited low activity towards butyrate and valerate (Table 3). This fraction was re-chromatographed on calcium phosphate gel using acetate as the substrate to

Table 3 Purification of acetyl CoA synthetase from bovine heart mitochondria
The procedure of Qureshi and Cook (1975) was used

Amount of heart tissue used = 4.39 Kg
Wet weight of mitochondria = 211 g

Fraction	Total protein mg	Total units C_2	Specific activity (units/mg protein)[a] C_2	C_3	C_4	C_5	Yield %	Fold purification
Mitochondrial suspension	15,130	469,030	32	25	-	-	100	1
Mitochondrial extract[b]	6,840	574,560	84	60	-	-	122	3
Ammonium sulfate precipitate	1,428	184,212	129	109	-	-	39	4
DEAE-23 cellulose	251	120,480	480	412	-	-	25	15
Calcium phosphate gel	13	36,387	2,799	2,463	-	-	7	90

[a] a unit is defined as the amount of enzyme which catalyzes the disappearance of 1 μmole of coenzyme A per minute.

[b] prepared by freezing and thawing the mitochondrial suspension three times.

locate enzyme activity eluting from the column. The enzyme eluted as one peak and after concentration using the Dia-flow cell was active on both acetate and propionate. On the basis of the properties of this enzyme, to be described, this fraction was designated acetyl CoA synthetase.

The chromatograms obtained using this purification procedure were identical to those obtained using the procedures worked out for the purification of the fatty acid activating enzymes of liver mitochondria (Figures 7 and 8) and so will not be repeated here.

The complete purification is shown in Table 3. A 90 fold purification was achieved. The purification procedure of Qureshi and Cook (1975) was therefore applicable to the purification of acetyl CoA synthetase from bovine heart mitochondria. The purified enzyme was stable for months in the absence of glycerol, at -20^0. The enzyme showed a tendencey to aggregate and bind to glass as demonstrated by the fact that after storage in glass containers at -20^o enzyme protein levels decreased.

Electrophoresis showed the presence of one band (Figure 9) indicating that the preparation was homogeneous. Electrophoresis followed by the PAS stain was positive indicating the presence of carbohdyrate residues (Figure 9). Electrophoresis in the presence of SDS also gave one band (Figure 9).

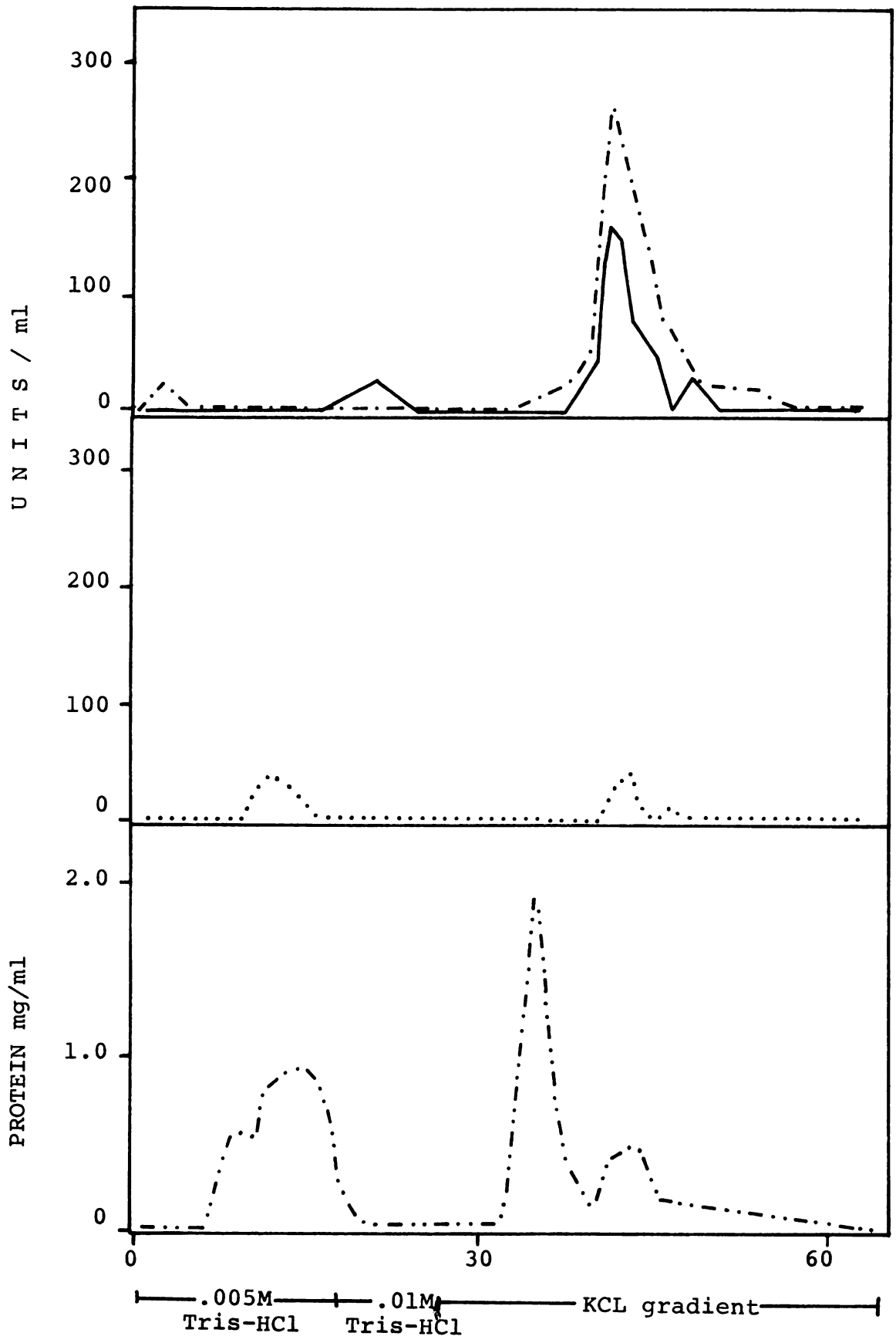
The apparent molecular weight was determined by SDS polyacrylamide electrophoresis (Figure 10) to be 73,000.

Figure 7 Chromatography of the fatty acid activating enzymes of heart mitochondria on DEAE-23 cellulose.

Column dimensions were 2 cm x 40 cm. The column was washed with 20 ml of 0.005 M Tris-HCl buffer pH 7.5 followed by 20 ml of 0.01 M Tris-HCl buffer pH 7.5. The activity was eluted with 100 ml of a linear KCl gradient of 0 to 0.6 M in 0.01 M Tris-HCl buffer, pH 7.5. All buffers contained 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was 40 ml/hr. The eluate was collected in 2.5 ml fractions. Collection tubes contained 0.25 ml glycerol.

-·-·- acetate activation
——— propionate activation
····· butyrate activation
-··-·· protein

a unit of enzyme activity is defined as the amount which catalyzes the disappearance of 1 μmole of coenzyme A per minute.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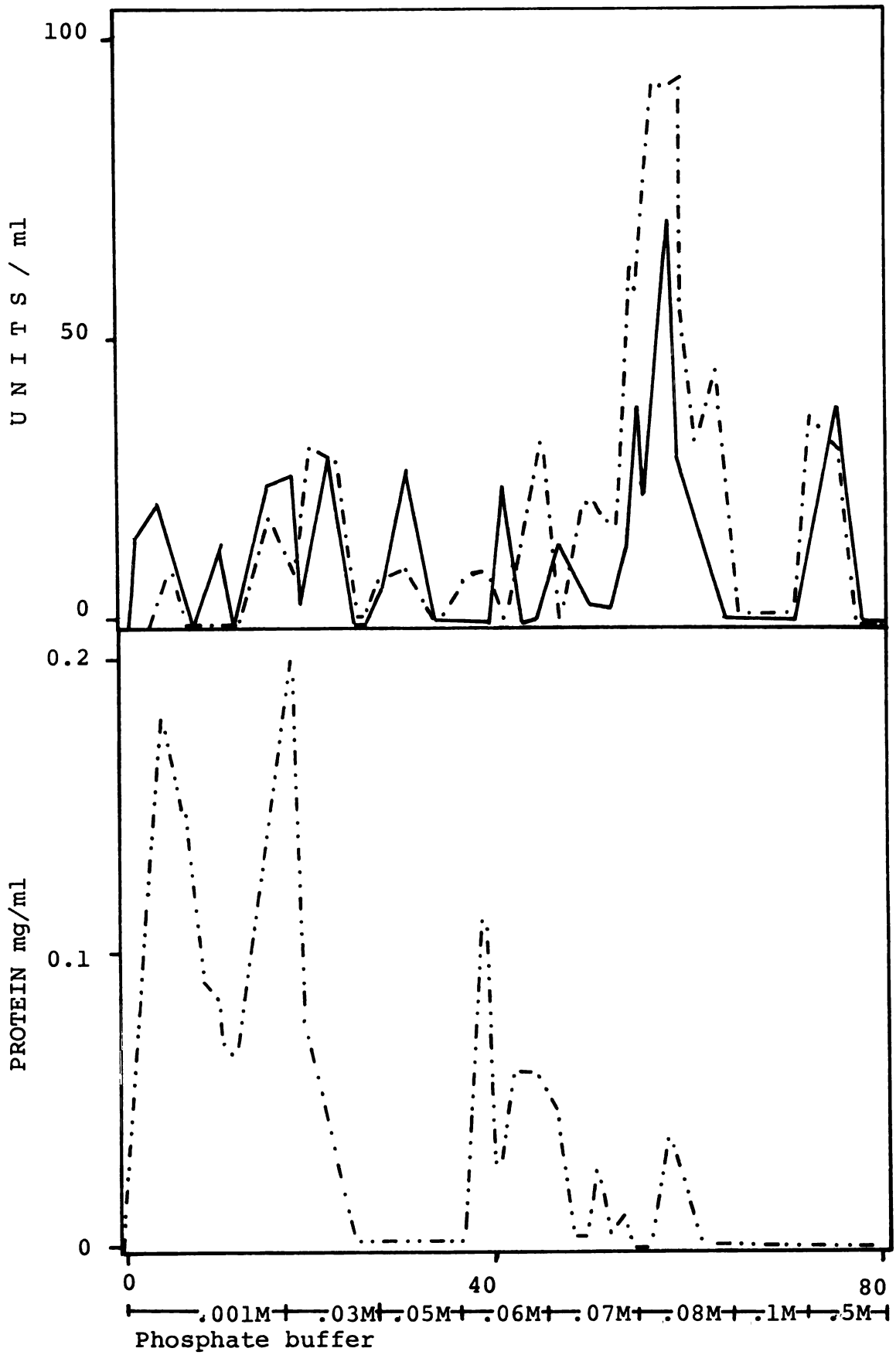
200
100
0
U N I T S / ml
300
200
100
0
2.0
1.0
0
PROTEIN mg/ml
0
30
60
.005M Tris-HCl
.01M Tris-HCl
KCL gradient
FRACTION NUMBER

Figure 8 Chromatography of acetyl CoA synthetase of heart mitochondria on calcium phosphate gel (using enzyme prepared from DEAE-23 cellulose chromatography Figure 7).

Column dimensions were 1.3 cm x 4.6 cm. The column was washed with a stepwise gradient on increasing concentration of potassium phosphate buffer, pH 7.0 in 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was 20ml/hr. The eluate was collected in 1 ml fractions. Collection tubes contained 0.1 ml glycerol.

—·—·— acetate activation
——— propionate activation
—··—·· protein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50
0
U N I T S / ml
0.2
0.1
0
PROTEIN mg/ml
0
40
80
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.03M
.05M
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.07M
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.1M
.5M
Phosphate buffer
FRACTION NUMBER

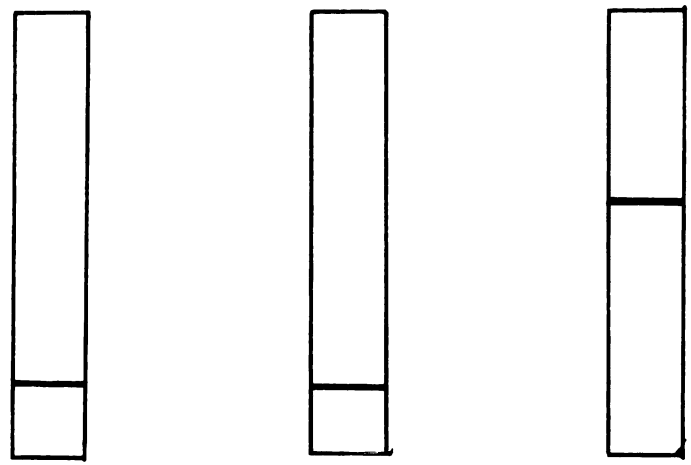


Figure 9 Schematic presentation of polyacrylamide gel electrophoresis of acetyl CoA synthetase purified from bovine heart mitochondria.

Details of the procedures used are given in the materials and methods section.

a gel stained with coomassie blue
b gel stained using the PAS procedure
c SDS gel stained with coomassie blue

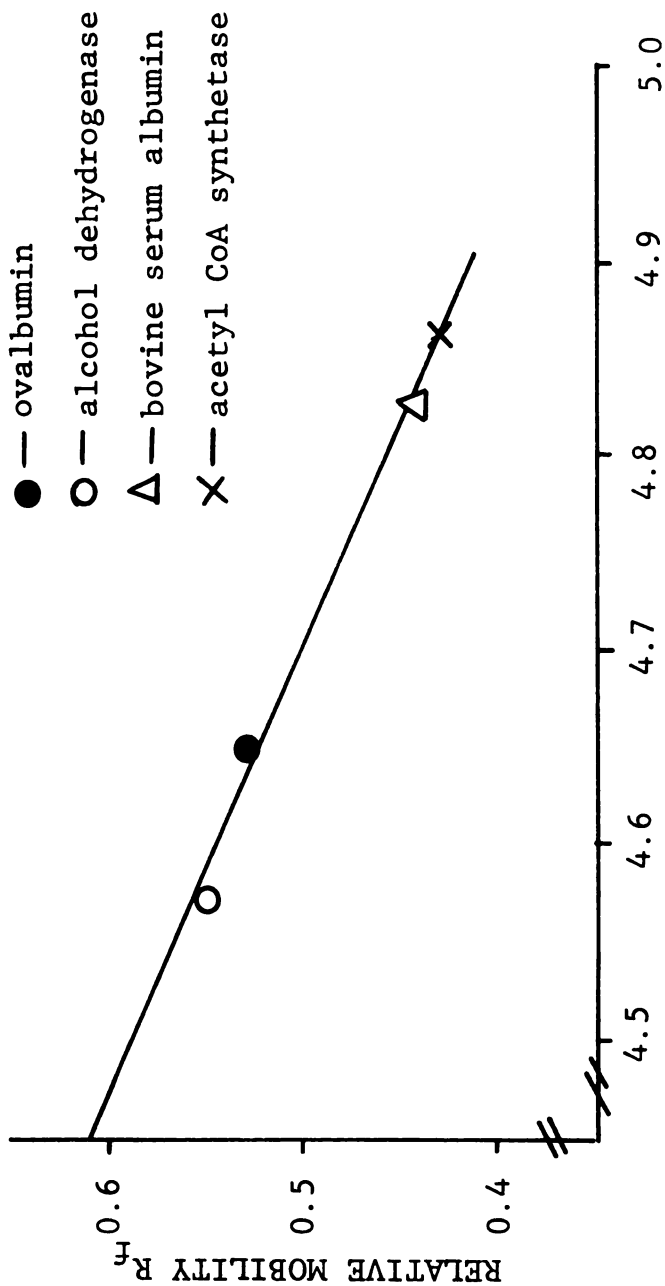


Figure 10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified acetyl CoA synthetase isolated from bovine heart mitochondria and standards of known molecular weight.

Details of the procedure used are given in the materials and methods section.

The molecular weight was also measured using the technique of sucrose density centrifugation. Both acetate and propionate were used as substrates to measure enzyme acitivity. Enzyme activity was associated with one peak (Figure 11) and this had an apparent molecular weight of 62,000. The data suggest that the enzyme is composed of one polypeptide chain of apparent molecular weight 67,500.

The amount of sialic acid was measured in each of three purified enzyme preparations. The sialic acid content of the three preparations was 0.79, 1.92 and 3.72 µg/mg protein respectively. Treatment with sialidase for 30 minutes at 37^{o} in acetate buffer at pH 6.0 and subsequent dialysis against Tris-HCl buffer pH 8.6 had no effect on enzyme activity when either acetate or propionate were used as substrates suggesting that the presence of sialic acid does not influence enzyme activity.

Gas liquid chromatography for detection of sugar residues was performed on six purified enzyme preparations. The results are summarized in Table 4. Mannose, galactose, glucose, n-acetyl galactosamine and n-acetyl glucosamine were found to be present.

Further studies on the heart enzyme were carried out using a different purification procedure. Subsequent work showed that isolation of liver and kidney acyl CoA synthetases could not be achieved using the purification procedure cited above. Procedures were developed for these tissues. In order that direct comparisons could be made

Figure 11 Sucrose density centrifugation of acetyl CoA synthetase of heart mitochondria.

Centrifugation was carried out at 50,000 rpm for 12 hours at 4° using a 5-20% sucrose gradient.

—·—·— acetate activation
——— propionate activation
—··—·· bovine serum albumin
— — · — — ovalbumin

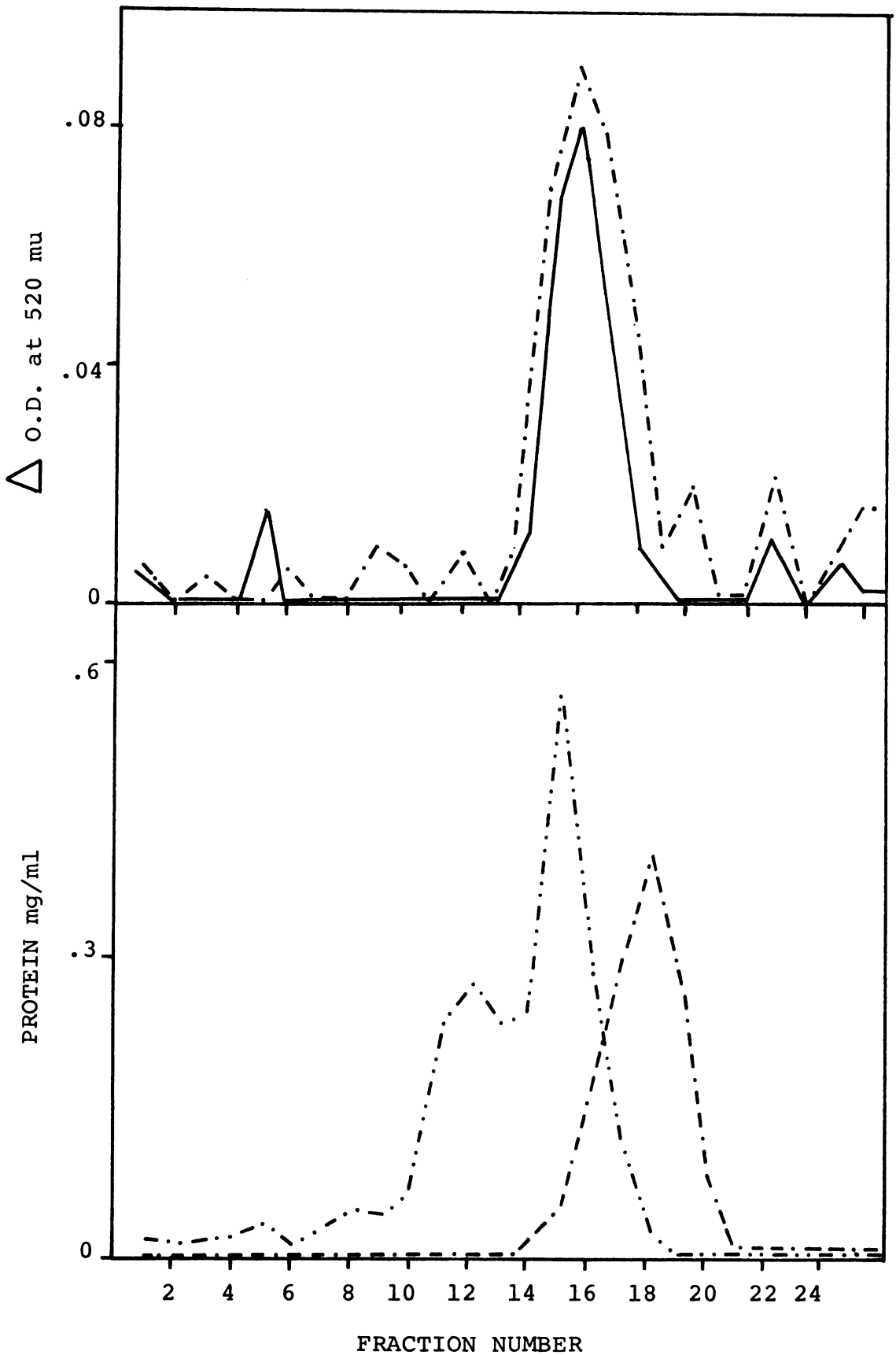

.08
.04
0
Δ O.D. at 520 mu
.6
.3
0
PROTEIN mg/ml
2
4
6
8
10
12
14
16
18
20
22
24
FRACTION NUMBER

Table 4 Carbohydrate content of acetyl CoA synthetase purified from bovine heart mitochondria.

Monosaccharide	µg/mg protein
D-mannose	5.2
D-galactose	8.3
D-glucose	2.5
N-acetyl-galactosamine	11.5
N-acetyl-glucosamine	3.0

samples were analyzed by gas liquid chromatography of the trimethylsilyl derivatives of methyl glycosides.

between heart, liver and kidney fatty acid activating enzymes, the final characterization of the acyl CoA synthetases from heart tissue was done using enzyme purified by the purification techniques worked out for the isolation of the enzymes from liver.

In order to determine how many short chain volatile fatty acid activating enzymes were present in heart mitochondrial tissue, acetate and propionate and in some cases butyrate and valerate, were used to monitor enzyme activity in column effluents.

Enzymes were liberated from mitochondria by sonication (Table 5). The mitochondrial extract showed maximal activity towards acetate followed by propionate while activity on butyrate and valerate was marginal (Table 5). Mitochondria were isolated and ammonium sulfate fractions prepared the same day. The ammonium sulfate precipitate was stored at -20^{o} until required for chromatography.

Column chromatography on DEAE-23 cellulose is shown in Figure 7. The enzymes could be eluted using a KCl gradient. Both acetate and propionate activating ability co-eluted as a single peak. Very little butyrate activation could be detected although a small peak eluted in the equilibration buffer at a point where the butyrate activation of liver and kidney mitochondrial tissue eluted (Figures 7, 19, 33). This probably represents the butyrl CoA synthetase purified by Webster et al. (1965). Some butyrate activation was associated with the major enzyme

Table 5 Purification of acetyl CoA synthetase from bovine heart mitochondria. The procedure developed for the purification of the fatty acid activating enzymes from liver was used.

Amount of heart tissue used = 740 g
Wet weight of mitochondria = 8.5 g

Fraction	Total protein mg	Total units C_2	Specific activity (units/mg protein)[a] C_2	C_3	C_4	C_5	Yield %	Fold purification
Mitochondrial suspension	798	9,490	12	11	8	11	100	1
Mitochondrial extract[b]	331	36,438	110	58	39	24	384	9
Ammonium sulfate precipitate	33	15,246	462	213	71	58	161	39
DEAE-23 cellulose	2.6	2,700	1,038	500	0	0	29	87
Calcium phosphate gel	0.06	573	9,550	4,000	0	0	6	796

[a] a unit is defined as the amount of enzyme which catalyzes the disappearance of 1 μmole of coenzyme A per minute

[b] prepared by sonciation of mitochondria

peak. The major enzyme peak was concentrated and applied to a calcium phosphate gel column (Figure 8). The enzyme activating acetate and propionate eluted in the 0.08 M potassium phosphate buffer. The concentrated enzyme activated primarily acetate followed by propionate with negligible activity on butyrate or valerate (Table 5).

The complete purification is shown in Table 5. A 793 fold purification was achieved. In the new procedure the enzyme did not denature as rapidly on concentration and thus higher specific activities were obtained.

The effect of acetate concentration on the activity of acetyl CoA synthetase is shown in Figure 12, The K_m was determed to be 1.79×10^{-4}M using the Lineweaver-Burk plot (r=.969) and 2.0×10^{-4}M using the Eadie-Scatchard plot (r=-.945). The effect of various other substrates on enzyme activity is shown in Table 6. Maximal activity was obtained using acrylate as a substrate followed by acetate and propionate. No activity could be detected using butyrate or valerate as substrates.

The effect of pH on enzyme activity is shown in Figure 13. The enzyme is active over a rather broad pH range from 6.0 to 11.3, the highest pH measured. No activity could be detected at or below pH 5.0. Acetyl CoA synthetase from mammary tissue precipitates and is inactivated at its iso-electric point (5.7) (Qureshi, 1971).

The effect of AMP on enzyme activity is shown in Figure 14. AMP is a weak inhibitor of acetyl CoA synthetase

Figure 12 Effect of acetate concentration on the activity of acetyl CoA synthetase purified from heart mitochondria.

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M-moles

Δ o.D.
0.10
0.05
0
0
10
20
30
[S](M) x 10^-3
v(M x min^-1 x mg^-1) x 10^-9
200
400
K_m=1.79 x 10^-4 M
v/[S] (min^-1 x mg^-1) x10^6
1000
500
0
0
1/[S]^2(M^-1) x 10^3
2
4
K_m=2.04 x 10^-4 M
1/v(M^-1 x min x mg) x10^9
.0050
.0025
0

Table 6 Substrate specificity of acetyl CoA synthetase purified from bovine heart mitochondria.

Substrates Tested	Relative Activity
Acetate	100
Propionate	67
Butyrate	0
Valerate	0
Hexanoate	0
Heptanoate	0
Octanoate	0
Acrylate	140
Maleate	0
Crotonate	19

Figure 13 Effect of pH on acyl CoA synthetase activity.

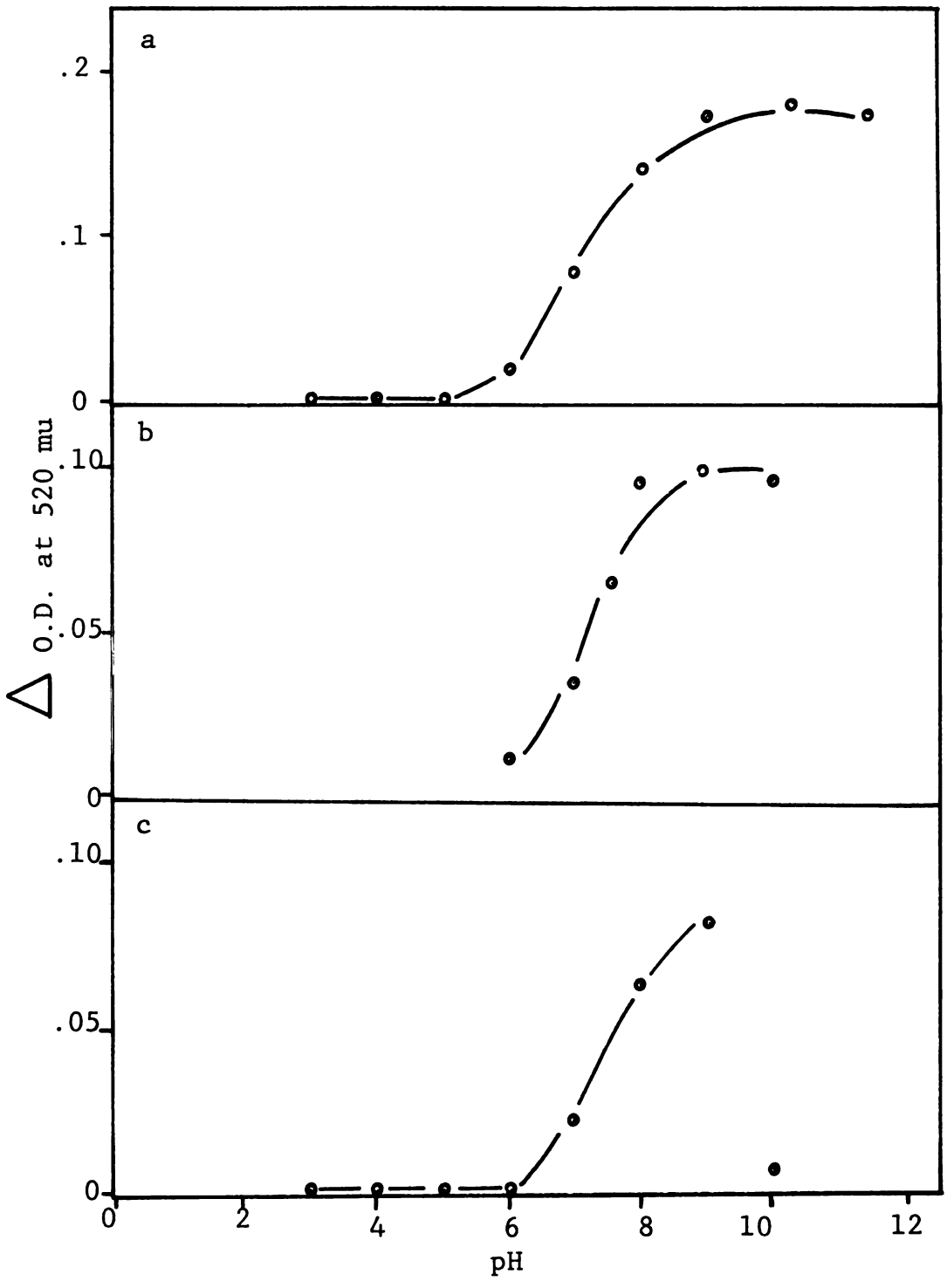
a. acetyl CoA synthetase purified from bovine heart mitochondria.

b. propionyl CoA synthetase purified from bovine liver mitochondria.

c. butyrate activating fraction isolated from bovine liver mitochondria.

pH 3.0 - 5.0 glycine-HCl buffer
6.0 - 7.0 phosphate buffer
8.0 - 9.0 Tris-HCl buffer
10.0 -11.4 glycine-NaOH buffer

Enzymes were preincubated in the appropriate buffer for one hour prior to assay.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b
c
.2
.1
0
.10
.05
0
.10
.05
0
Δ O.D. at 520 mu
0
2
4
6
8
10
12
pH

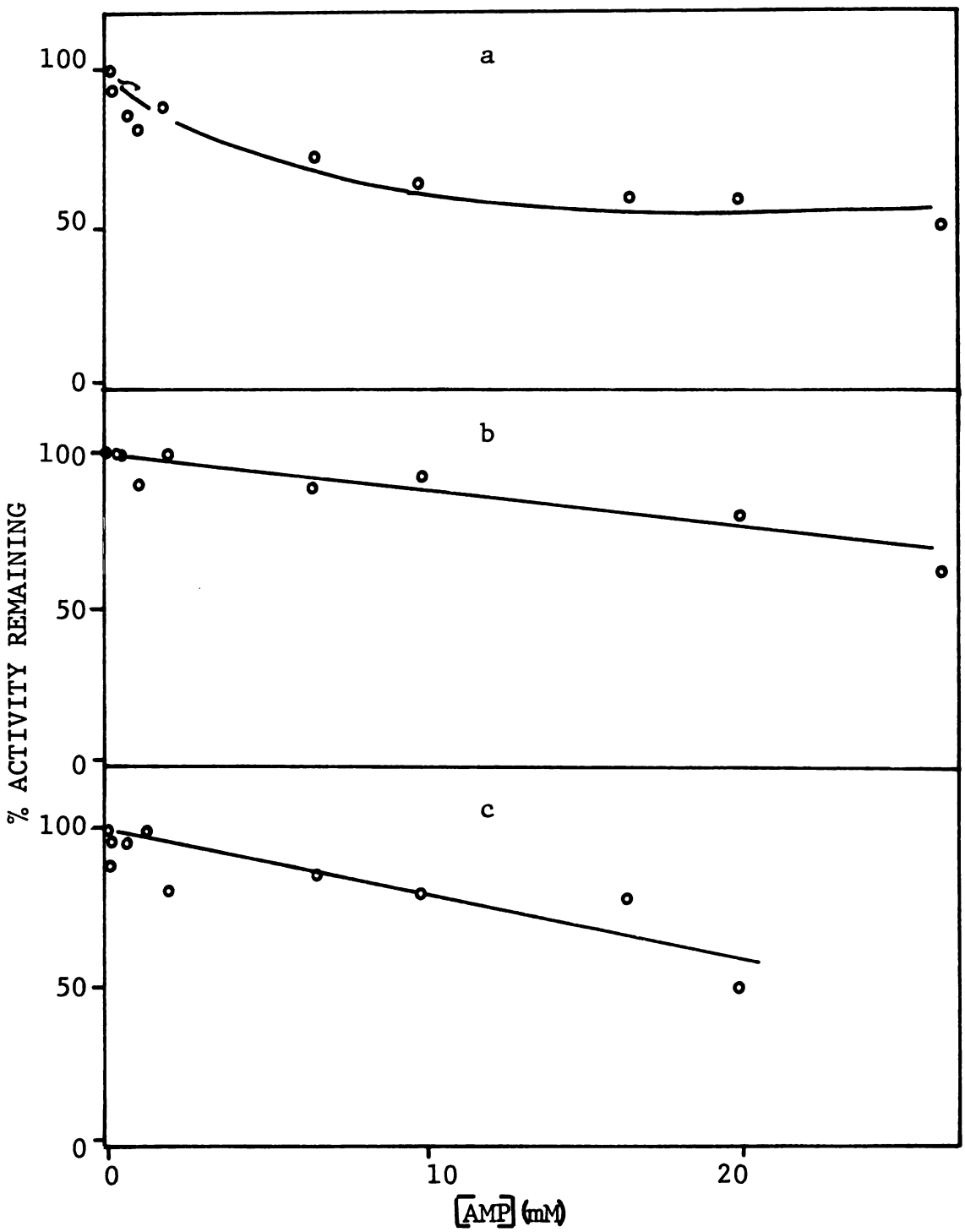
Figure 14 Effect of AMP concentration on acyl CoA synthetase activity.

a. acetyl CoA synthetase purified from bovine heart mitochondria.

b. propionyl CoA synthetase purified from bovine liver mitochondria.

c. butyrate activating fraction isolated from bovine liver mitochondria.

Enzymes were preincubated in AMP for 1 minute prior to assay.


a
b
c
100
50
0
% ACTIVITY REMAINING
0
10
20
[AMP] (mM)

(K_i=1.13x10^{-4}M).

During the search for additional techniques with which to purify the enzymes from liver and kidney tissue it was found that affinity chromatography using 5'-AMP-Sepharose 4B and hydrophobic chromatography using Agarose with different lengths of alkyl side chains could be used. These are discussed below for comparitive purposes.

The chromatography of the ammonium sulfate precipitate on 5'-AMP-Sepharose 4B is shown in Figure 15. The enzyme did not bind to the affinity column. However, a substantial purification was achieved since large quantities of non-enzyme protein did bind to the column. One anomaly was observed. There appeared to be a substantial loss of acetate activating ability on the column. Although the enzyme is normally more active on acetate than on propionate, fractions eluting from the column contained greater amounts of propionate activating ability than acetate. On concentration however the enzyme reverted to the normal pattern of activation. The reason for this phenomenon is not clear.

Hydrophobic chromatography of acetyl CoA synthetase is shown in Figure 16. Ammonium sulfate precipitate was used. With increasing lengths of alkyl side chain greater quantities of enzyme were bound. However, with alkyl chains of eight carbon units or greater enzyme protein bound so tightly that it could not be eluted with 0.001 M potassium phosphate buffer. The best recovery of protein was 60% and the greatest increase in specific activity was from 32 to

Figure 15 Chromatography of the fatty acid activating enzymes of heart mitochondria on 5'-AMP-Sepharose 4B.

Column dimensions were 0.6 cm x 20 cm. 25 mg of enzyme protein was applied to the column. This was washed onto the column with 32 ml of 0.001 M potassium phosphate buffer pH 7.0. Non-enzyme protein was eluted with 0.6 M KCl pH 7.0. Flow rate was 15 ml/hr. The eluate was collected in 1.6 ml fractions. All buffers contained 10% glycerol and 2.5 mM 2-mercaptoethanol.

-·-·- acetate activation
——— propionate activation
-··-·· protein

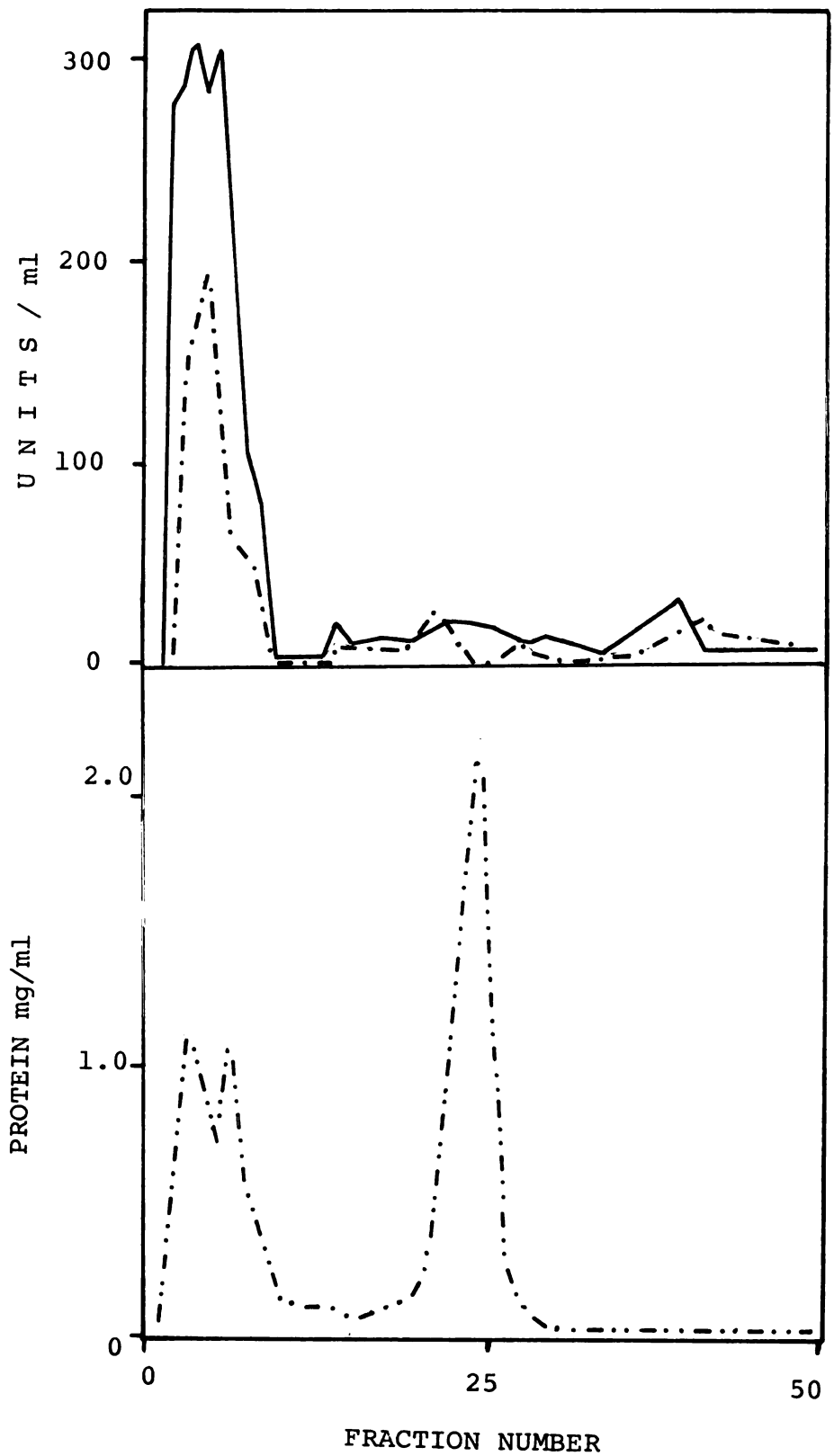
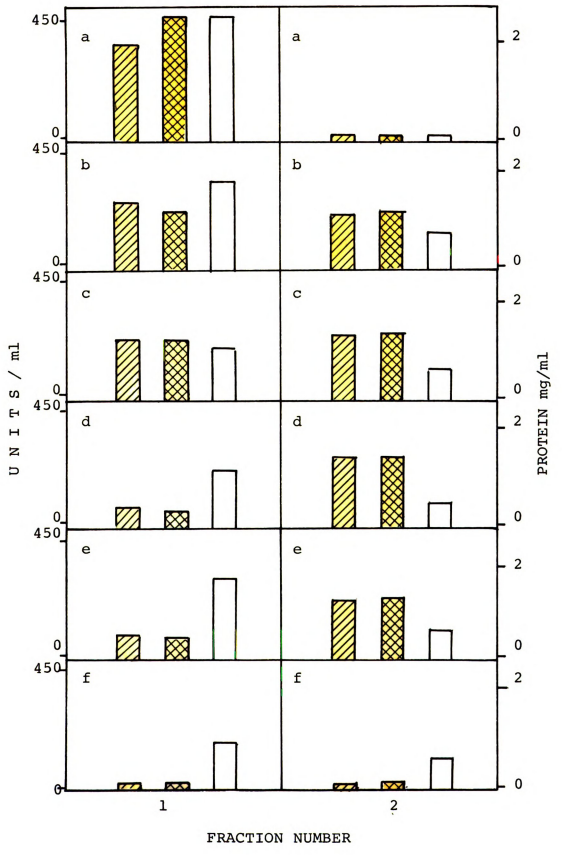

300
200
100
0
U N I T S / ml
2.0
1.0
0
PROTEIN mg/ml
0
25
50
FRACTION NUMBER

Figure 16 Hydrophobic chromatography of the fatty acid activating enzymes of heart mitochondria.

4.5 mg of protein was placed on each of the six columns (a-f). The protein was washed onto each column with 2.5 ml of 1 M potassium phosphate buffer pH 7.5. The eluate collected from each column was collected into one tube and designated fraction 1. Each column was then eluted with 2.5 ml of 0.05 M potassium phosphate buffer pH 7.5 containing 10% glycerol and 2.5 mM 2-mercaptoethanol. The eluate was collected into one tube and designated fraction 2.

a. - Agarose
b. - Agarose - CH_2-CH_3
c. - Agarose - $(CH_2)_3$-CH_3
d. - Agarose - $(CH_2)_5$-CH_3
e. - Agarose - $(CH_2)_7$-CH_3
f. - Agarose - $(CH_2)_9$-CH_3


450
0
a
b
c
d
e
f
2
0
UNITS / ml
PROTEIN mg/ml
1
2
FRACTION NUMBER

500 mμmoles/min/mg protein.

Purification and Characterization of the Fatty Acid Activating Enzymes of Bovine Liver Mitochondria

Preliminary studies were conducted using mitochondrial suspensions prepared by the method of Qureshi and Cook (1975). Preparations were obtained from livers of different animals and these showed large variations in the amount of fatty acid activating ability. The presence of activity in some preparations prior to mitochondrial rupture suggested that these enzymes are located on the outside of the mitochondrial surface (Table 7). Liberation of the enzymes from the mitochondrial membranes by the freezing and thawing process of Qureshi and Cook (1975) resulted in complete loss of enzyme activity. Enzyme preparations allowed to stand overnight at 0^{o} or 20^{o} also lost activity. However, subsequent work has demonstrated that the enzymes are not cold labile.

A search was initiated therefore for methods by which the enzymes could be both stabilized and isolated from the mitochondrial membranes for subsequent purification.

Some enzyme activity could be retained using the acetone powder technique of Mahler et al.(1953) to achieve mitochondrial rupture. Providing the mitochondrial extract thus obtained was immediately subjected to fractionation with ammonium sulfate a stable preparation could be obtained. However, all enzyme activity was lost when the preparations were subjected to subsequent column

Table 7 Purification of propionyl CoA synthetase from bovine liver mitochondria.

Amount of liver tissue used = 100 g
Wet weight of mitochondria = 14 g

Fraction	Total protein	Total units	Specific activity (units/mg protein)[a]			Yield %	Fold purification
		C_3	C_2	C_3	C_4	C_5		
Mitochondrial suspension	1,477	47,264	8	32	29	28	100	1
Mitochondrial extract[b]	628	35,185	10	56	37	30	74	2
Ammonium sulfate precipitate	241	29,631	12	123	62	47	63	4
DEAE-23 cellulose	50	18,920	30	378	97	90	40	12
Calcium phosphate gel First peak	11	1,000	15	100	386	438		
Second peak	1.6	5,809	195	3,631	208	76	14	114

[a] a unit is defined as the amount of enzyme which catalyzes the disappearance of 1 μmole of coenzyme A per minute

[b] prepared by sonciation of mitochondria

chromatography on either DEAE-23 cellulose or calcium phosphate gel.

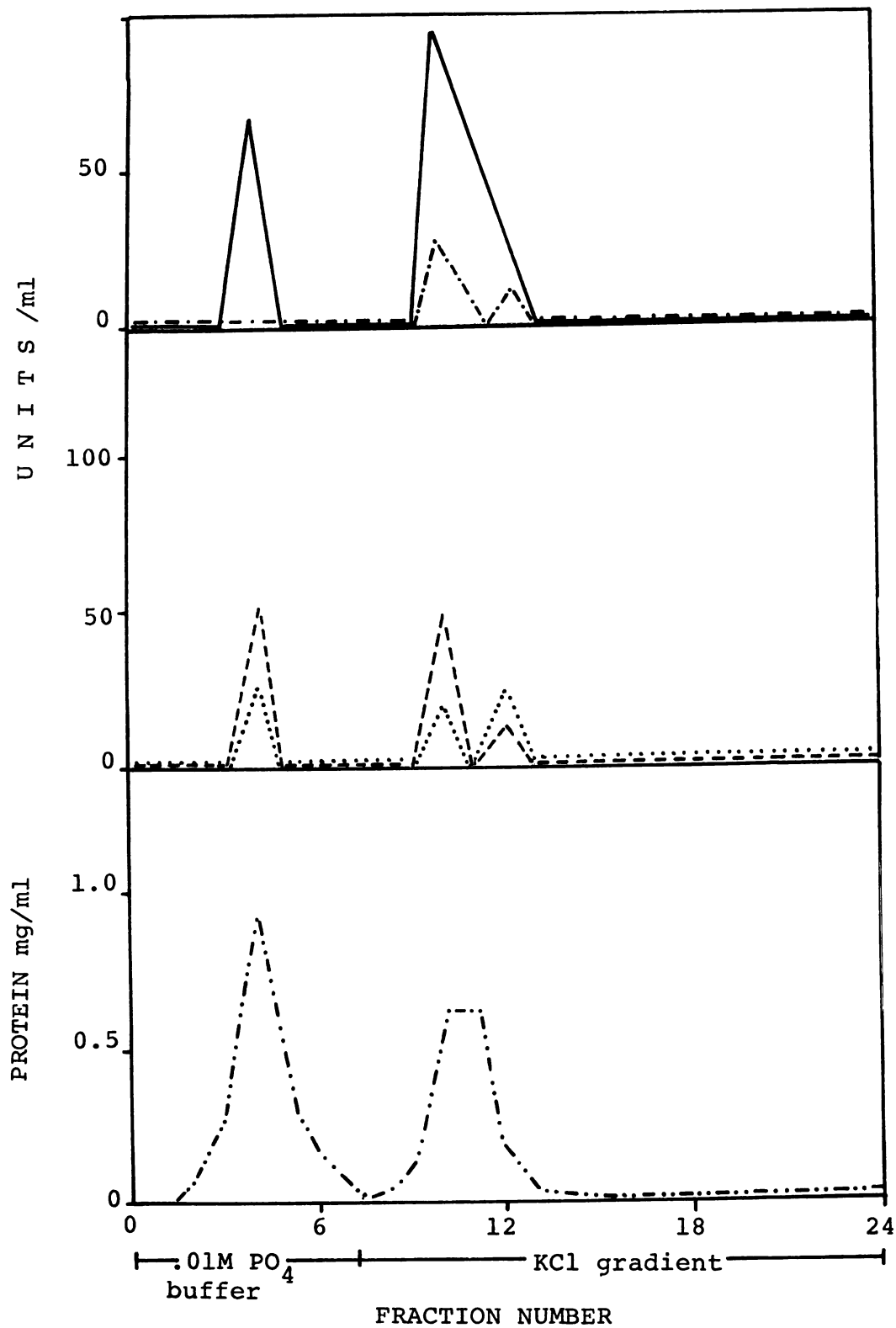
Satisfactory purifications could be achieved by using 10% glycerol in the buffer used to suspend the mitochondria. The enzymes appear to be stable in the presence of 10% glycerol. The presence of glycerol prevents rupture of mitochondria by freezing and thawing. Mitochondrial rupture was achieved by sonication as described in the materials and methods section. Enzyme activity could be maintained only if all purification steps were carried out in the presence of glycerol.

A variety of column chromatography techniques were investigated in an effort to isolate the fatty acid activating enzymes from liver. The cation exchanger phosphocellulose had been used effectively to separate the fatty acid activating enzymes of guinea pig liver mitochondria (Groot, 1976). Separation using this support is shown in Figure 17. Two enzymes could be separated. The first enzyme, about equally active on propionate and butyrate, did not bind to the support and eluted in the void volume. No increase in specific activity was observed. The second enzyme could be eluted by a KCl gradient. This enzyme exhibited maximal activity towards propionate although some activity was obtained using butyrate or valerate as substrates to measure enzyme activity. Very little increase in specific activity was observed and since a main objective was to isolate a propionyl CoA synthetase this

Figure 17 Chromatography of the fatty acid activating enzymes of liver mitochondria on phosphocellulose.

Column dimensions were 1.2 cm x 20 cm. 112 mg of protein was applied to the column. This was washed on with 50 ml of 0.01 M potassium phosphate buffer containing 10% glycerol and 2.5 mM 2-mercaptoethanol, pH 6.5 and then eluted with 200 ml of a linear KCl gradient of 0 to 2.0 M in the same buffer. The eluate was collected in 10.3 ml fractions. Each collection tube contained 1 ml of glycerol. Flow rate was 25 ml/hr.

—·—·— acetate activation
——— propionate activation
····· butyrate activation
— — — valerate activation
—··—·· protein


50
0
U N I T S /ml
100
50
0
1.0
0.5
0
PROTEIN mg/ml
0
6
12
18
24
.01M PO4 buffer
KCl gradient
FRACTION NUMBER

method was rejected.

Affinity chromatography can allow the purification of an enzyme in one step. Moreover, it has the added advantage that while the enzyme is bound to the column support it generally cannot be inactivated. Since AMP was known to be a weak inhibitor of acetyl CoA synthetase isolated from bovine mammary mitochondria (Qureshi, 1971) and from bovine heart mitochondria (Figure 14) it seemed possible that chromatography on 5'-AMP-Sepharose 4B might be an effective way to purify these enzymes from liver. Chromatography of the ammonium sulfate fraction on this support is shown in Figure 18. Again two enzymes could be distinquished. The first enzyme which eluted in the 0.001 M potassium phosphate buffer was about equally active on propionate and butyrate and coeluted with the main protein peak. The second enzyme could be eluted with 0.1 M potassium chloride buffer and exhibited maximal activity towards propionate. A ten fold increase in specific activity of the second enzyme was achieved and 100 % of the protein applied to the column was recovered.

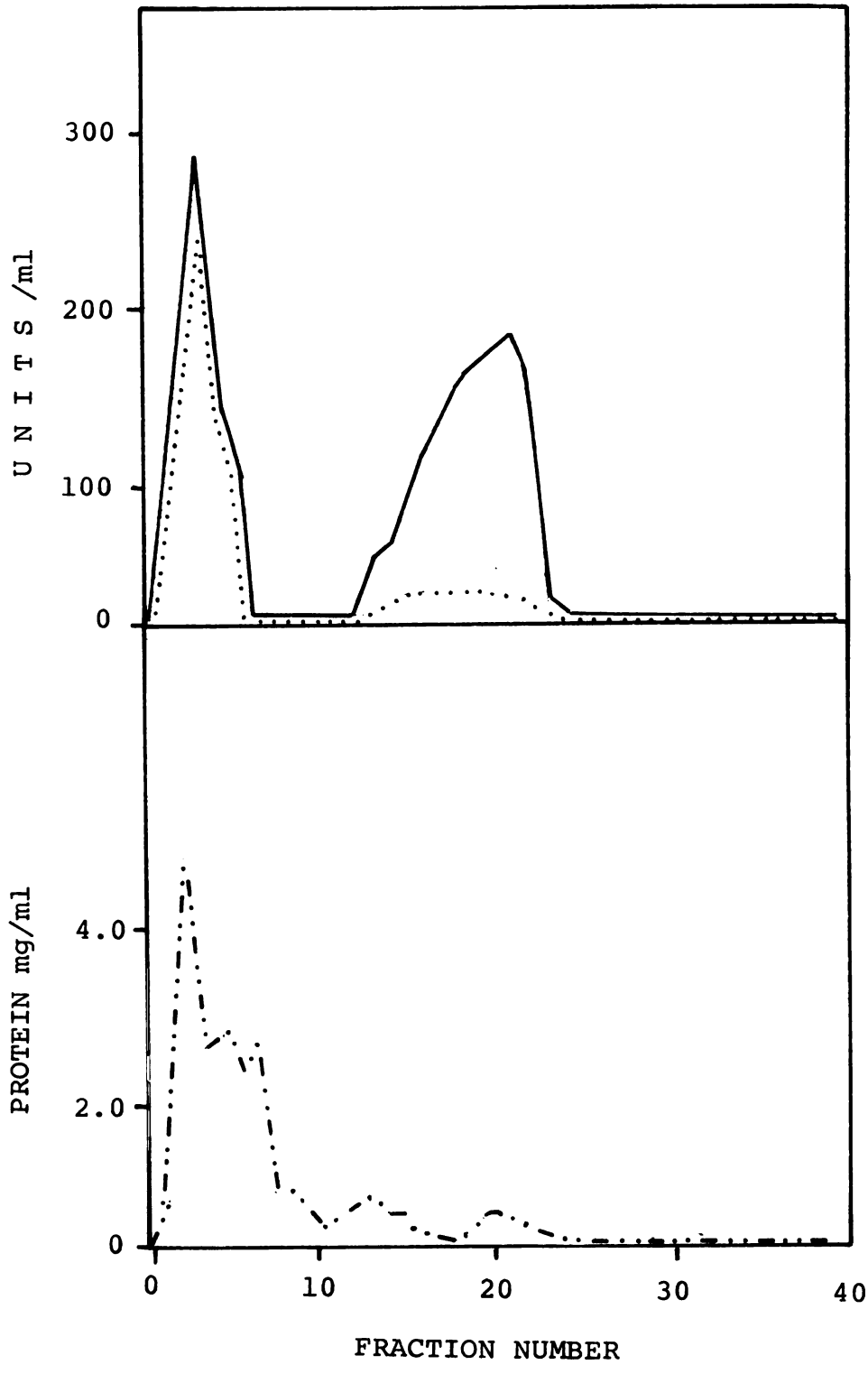
Hydrophobic chromatography of ammonium sulfate fraction resulted in substantial losses of fatty acid activating ability. This method was therefore rejected as unsuitable for purification of these enzymes.

Many unsuccessful attempts were made to purify these enzymes using DEAE-23 cellulose and calcium phosphate gel; techniques which were successful in purifying

Figure 18 Chromatography of the fatty acid activating enzymes of liver mitochondria on 5'-AMP-Sepharose 4B.

Column dimensions were 0.6 cm x 20 cm. 25 mg of protein was applied to the column. This was washed on with 20 ml of 0.001 M potassium phosphate buffer pH 7.0 followed by 20 ml of 0.01 M potassium phosphate buffer 7.0. The column was eluted with 20 ml of 0.1 M KCl followed by 20 ml of 0.6 M KCl both in 0.01 M potassium phosphate buffer pH 7.0. Flow rate was 15 ml/hr. The eluate was collected in 1.35 ml fractions. Collection tubes contained 0.15 ml glycerol. All buffers contained 10% glycerol and 2.5 mM 2-mercaptoethanol.

——— propionate activation
..... butyrate activation
—·—·— protein


300
200
100
0
U N I T S /ml
4.0
2.0
0
PROTEIN mg/ml
0
10
20
30
40
FRACTION NUMBER

acetyl CoA synthetase from heart mitochondria. However, by increasing the amount of protein added to a given column, by carefully maintaining glycerol concentration between 10-15% in all elution buffers and placing glycerol in the collection tubes such that the final concentration of glycerol in each fraction was 10-15% substantial progress in enzyme purification using these techniques could be made.

Although the enzymes did not bind to DEAE-23 cellulose substantial purification could be achieved by passage through this support (Figure 19). Activity was associated with a single peak; propionate, butyrate, valerate but not acetate being activated. After concentration of the tubes which contained fatty acid activating ability, the sample was applied to a calcium phosphate gel column and enzyme activity monitored using propionate, butyrate and valerate as substrates. The chromatogram (Figure 20) shows that the activity could be separated into two components; one which activated primarily butyrate and valerate but with some propionate activating ability and one which activated only propionate. Based on evidence to be presented later tha former fraction will be designated the butyrate activating fraction and the latter propionyl CoA synthetase.

The complete purification of the two enzymes from liver mitochondria is shown in Table 7. The mitochondrial extract can activate propionate, butyrate, and valerate

Figure 19 Chromatography of the fatty acid activating enzymes of liver mitochondria on DEAE-23 cellulose.

Column dimensions were 2.5 cm x 47 cm. Protein was applied to the column. The column was washed with 350 ml of 0.005 M Tris-HCl buffer pH 7.5. Enzyme activity was eluted with 700 ml of 0.01 M Tris-HCl buffer pH 7.5. Non-enzyme protein was eluted with 500 ml of a linear KCl gradient of 0 to 0.6 M in 0.01 M Tris-HCl buffer pH 7.5. All buffers contained 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was 60 ml/hr. The eluate was collected in 10 ml fractions. Collection tubes contained 1 ml glycerol.

——— propionate activation
..... butyrate activation
– – – – valerate activation
–··–·· protein

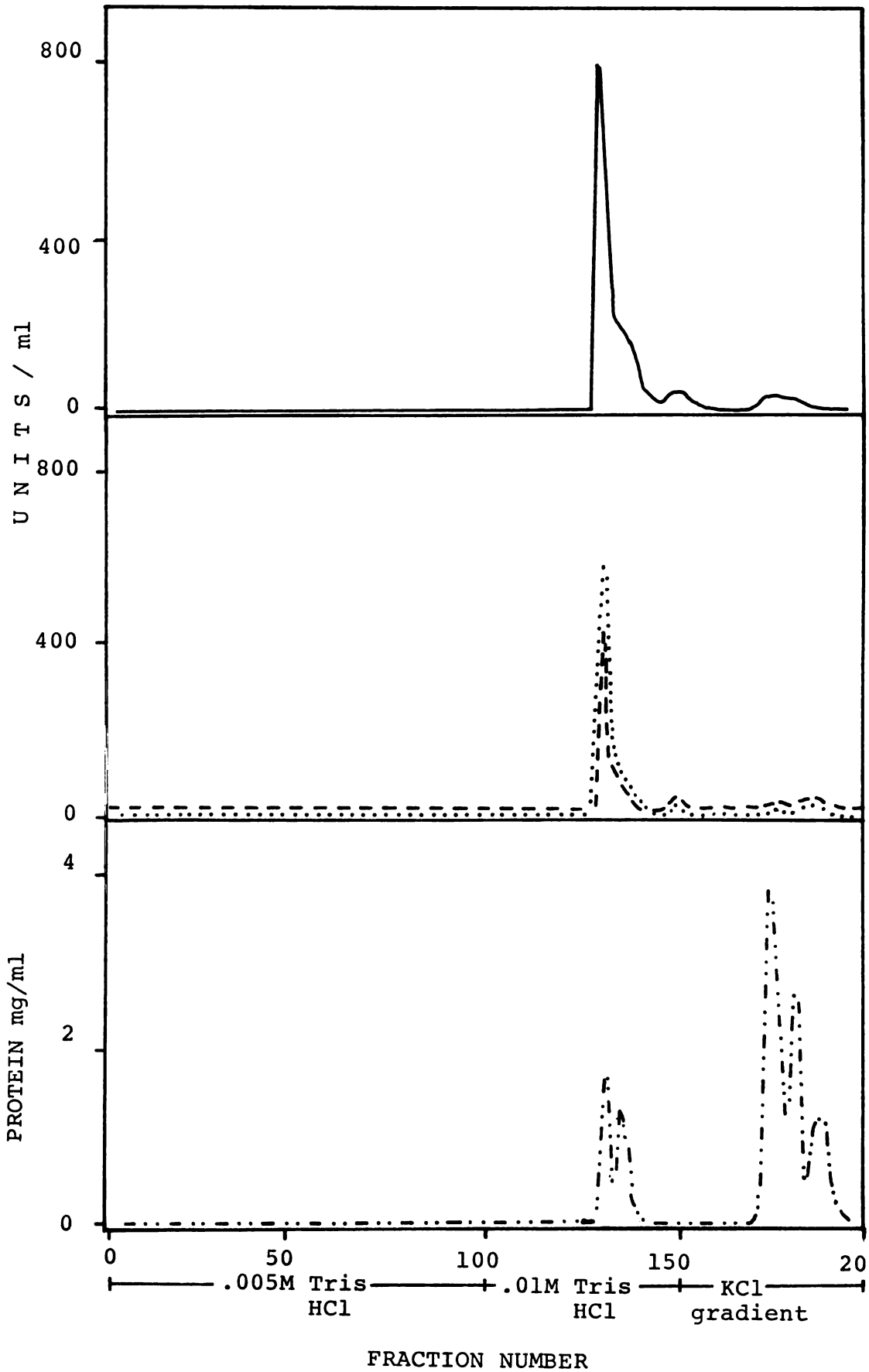
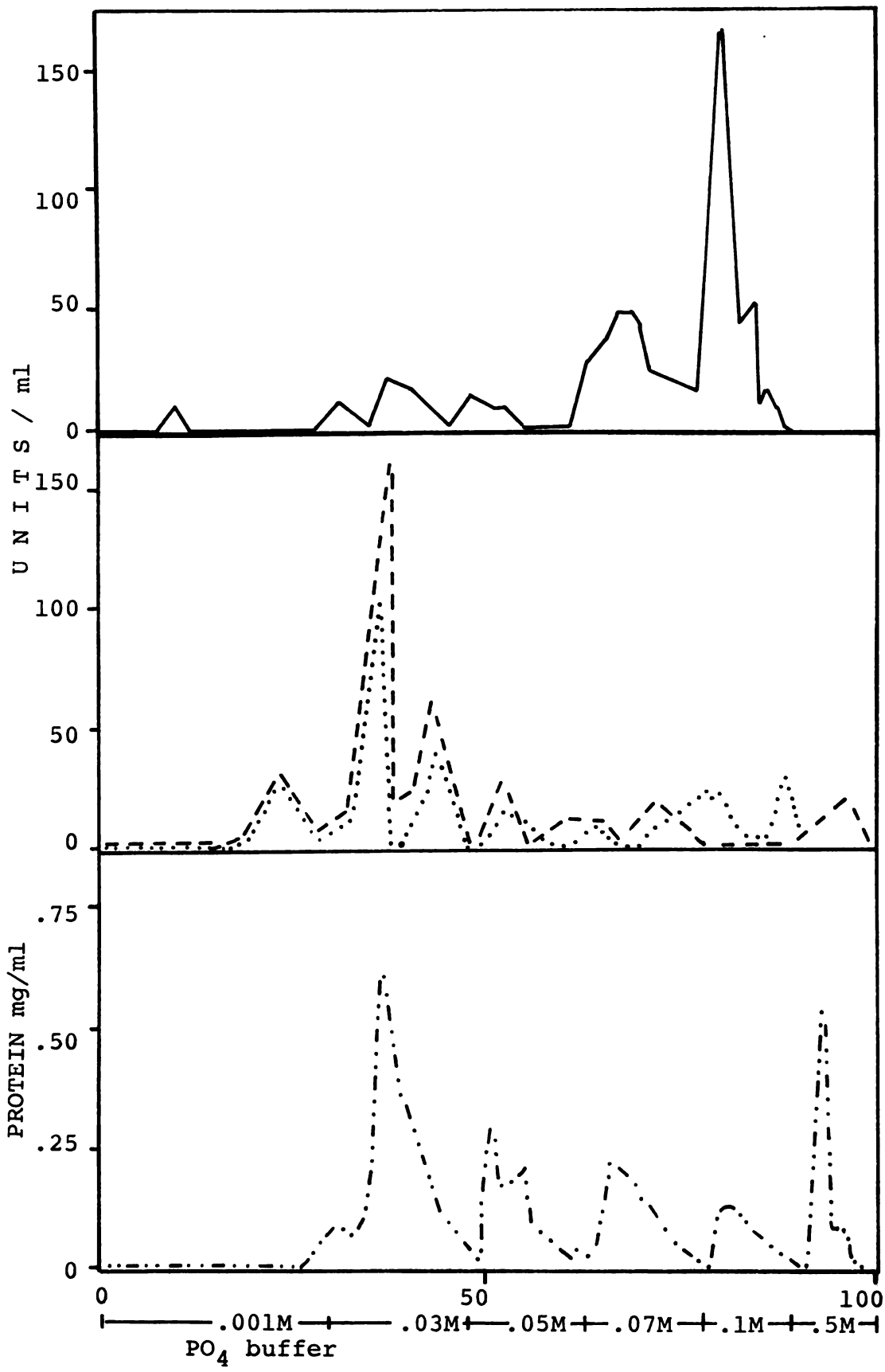

800
400
0
U N I T S / ml
800
400
0
4
2
0
PROTEIN mg/ml
0
50
100
150
200
.005M Tris HCl
.01M Tris HCl
KCl gradient
FRACTION NUMBER

Figure 20 Chromatography of the fatty acid activating enzymes of liver mitochondria on calcium phosphate gel (L fraction prepared from chromatography on DEAE-23 cellulose Figure 19).

Column dimensions were 3 cm x 15 cm. Protein was applied to the column. The column was washed with a stepwise gradient of increasing concentration of potassium phosphate buffer pH 7.0 in 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was 120 ml/hr. Each collection tube contained 1 ml glycerol. The eluate was collected in 10.3 ml fractions.

——— propionate activation
····· butyrate activation
– – – – valerate activation
—··—·· protein


150
100
50
0
U N I T S / ml
150
100
50
0
.75
.50
.25
0
PROTEIN mg/ml
0
50
100
.001M
.03M
.05M
.07M
.1M
.5M
PO4 buffer
FRACTION NUMBER

but has only marginal ability to activate acetate (Table 7). Using DEAE-23 cellulose and calcium phosphate gel chromatography a 111 fold purification of propionyl CoA synthetase was achieved. Both enzymes after concentration were stable for months if stored in the presence of 10% glycerol at -20°.

Electrophoresis of propionyl CoA synthetase (Figure 21) showed the presence of one major band and several minor bands. The same pattern was obtained on gels of different percent acrylamide. However it is not known whether enzyme activity was associated with the major band or with one of the minor bands. Electrophoresis of the butyrate activating fraction showed the presence of at least five major bands (Figure 22) indicating the heterogeneity of the preparation.

Molecular weight determinations were made for both enzymes using the technique of sucrose density centrifugation (Figures 23 and 24). The apparent molecular weight of propionyl CoA synthetase was determined to be 73,400. Sucrose density centrifugation of the butyrate activating fraction (Figure 24) indicated the presence of two enzymes with fatty acid activating properties; a butyryl CoA synthetase of apparent molecular weight 67,000 and a valeryl CoA synthetase of apparent molecular weight 65,000.

Sodium dodecyl sulfate polyacrylamide electrophoresis of propionyl CoA synthetase (Figure 25) gave 6 minor bands and one major band. The molecular weights of the proteins composing the minor bands were 68,000, 60,000, 57,000, 50,000, 45,000, 40,000 and the molecular weight of the protein composing the major band was 35,000. Since minor impurities

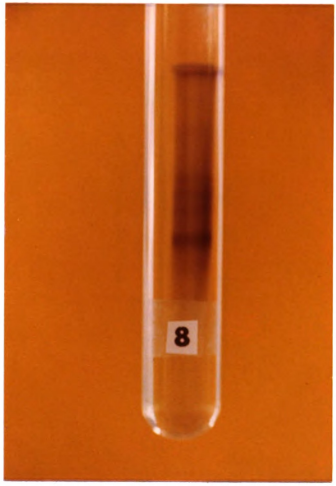


Figure 21 Polyacrylamide gel electrophoresis of propionyl CoA synthetase prepared from liver mitochondria.

A pH 8.3 buffer system was used. 200 ug of protein were layered on top of the gel. The run was carried for 6 hours at 3 ma/gel. The gel was stained with coomassie blue.

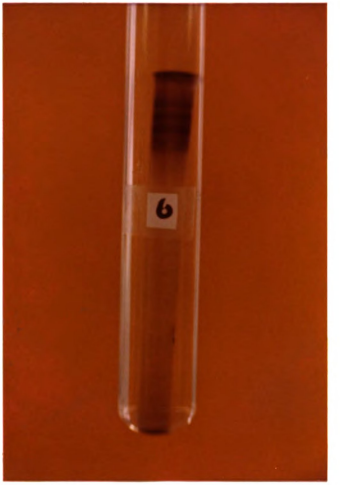


Figure 22 Polyacrylamide gel electrophoresis of the butyrate activating fraction of liver mitochondria.

A pH 8.3 buffer system was used. 75 ug of protein were layered on top of the gel. The run was carried out for 2 hours using 3 ma/gel. The gel was stained using coomassie blue.

Figure 23 Sucrose density centrifugation of propionyl CoA synthetase of liver mitochondria.

Centrifugation was carried out at 50,000 rpm for 12 hours at 4° using a 5-20% sucrose gradient.

——— propionate activation
····· butyrate activation
—··—·· bovine serum albumin
— — · — — ovalbumin

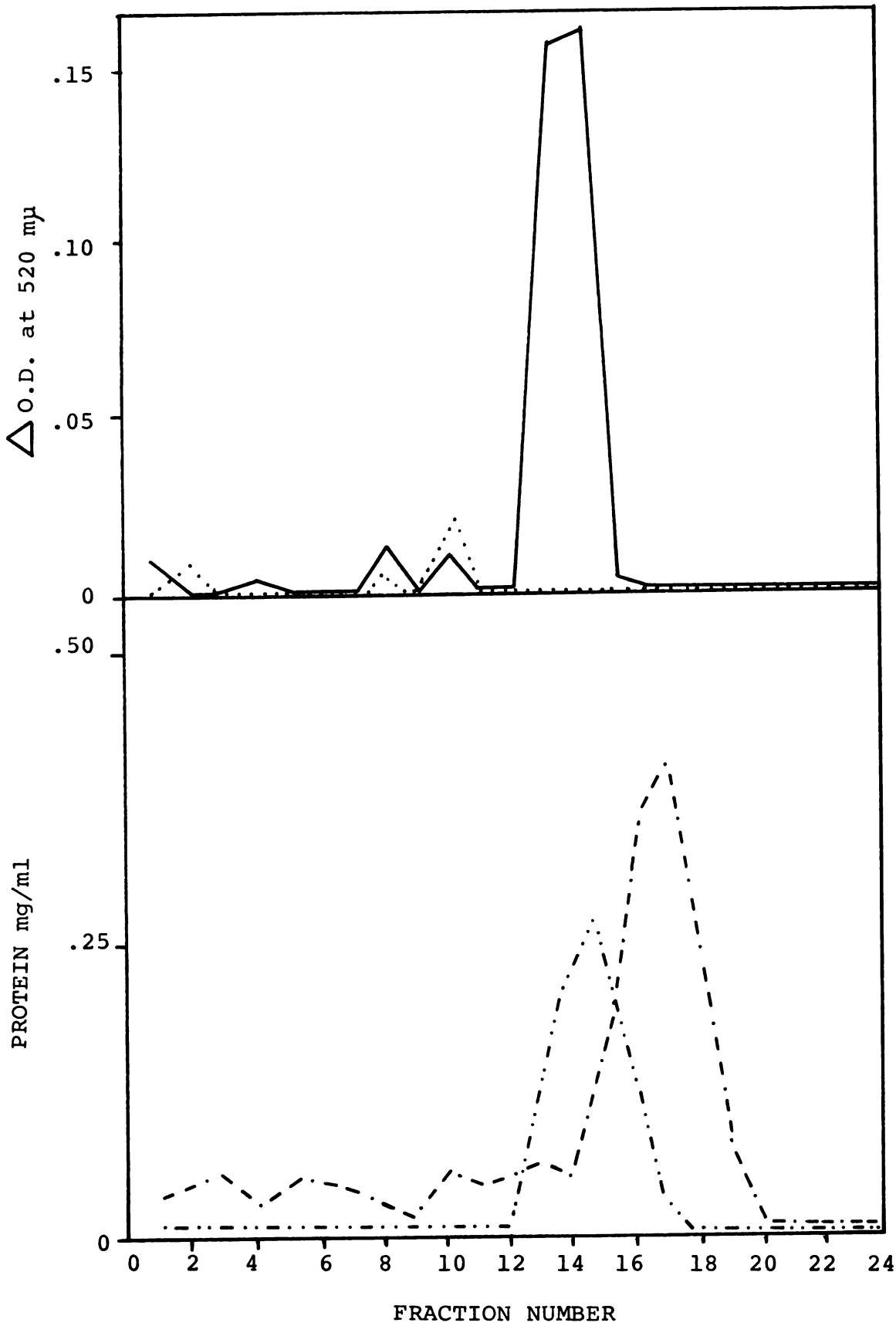
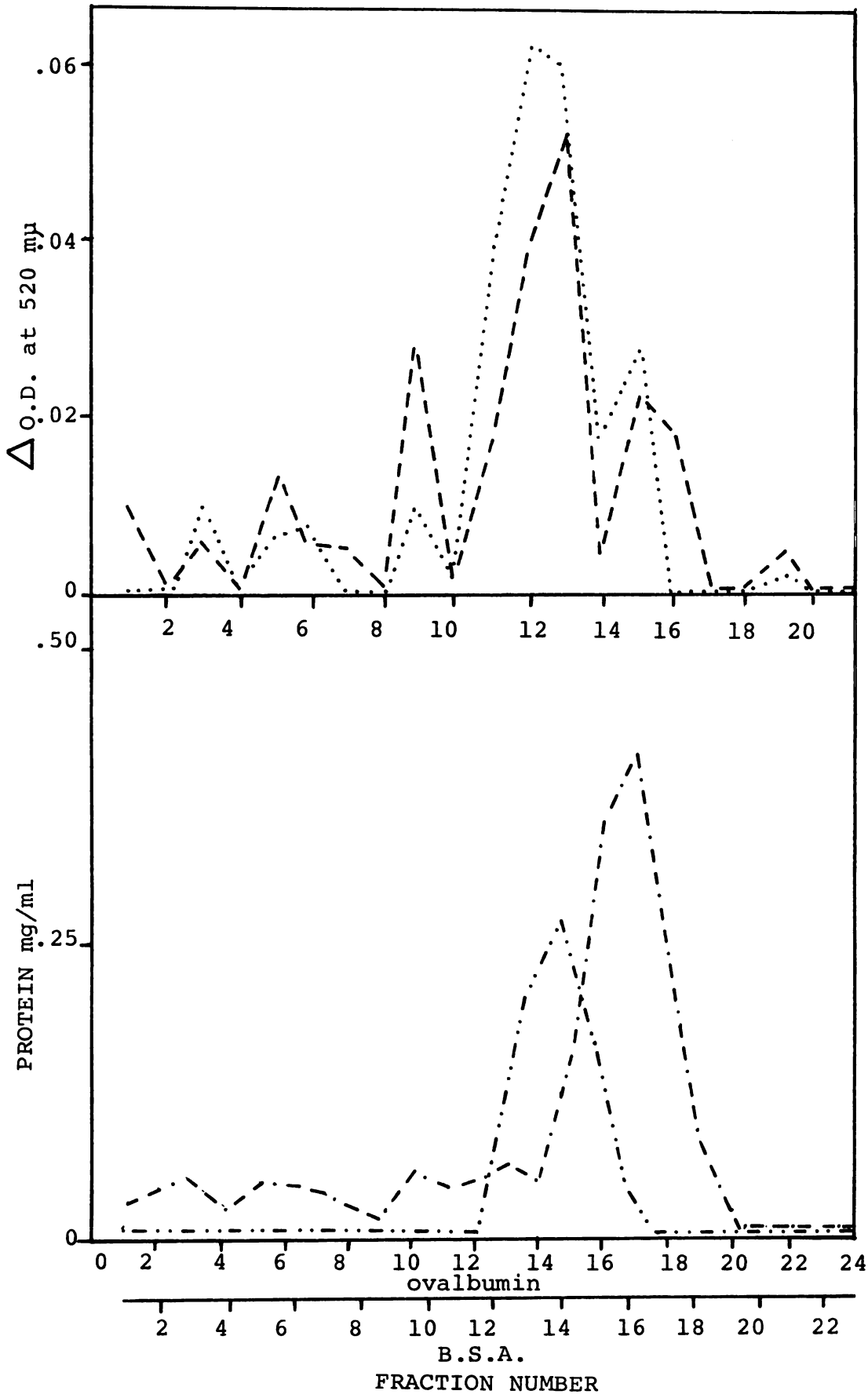

.15
.10
.05
0
Δ O.D. at 520 mμ
.50
.25
0
PROTEIN mg/ml
0
2
4
6
8
10
12
14
16
18
20
22
24
FRACTION NUMBER

Figure 24 Sucrose density centrifugation of the butyrate activating fraction of liver mitochondria.

Centrifugation was carried out at 50,000 rpm for 12 hours at 4° using a 5-20% sucrose gradient.

·····	butyrate activation
— — — —	valerate activation
—··—··	bovine serum albumin
- - · — —	ovalbumin


.06
.04
.02
0
Δ O.D. at 520 mμ
2 4 6 8 10 12 14 16 18 20
.50
.25
0
PROTEIN mg/ml
0 2 4 6 8 10 12 14 16 18 20 22 24
ovalbumin
2 4 6 8 10 12 14 16 18 20 22
B.S.A.
FRACTION NUMBER

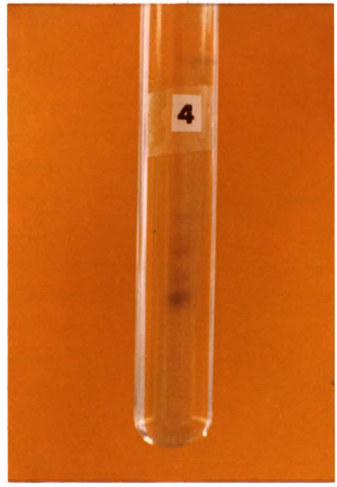


Figure 25 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of propionyl CoA synthetase prepared from liver mitochondria.

15 ug of protein was applied to the top of the gel. The run was carried out for 5 hours at 3 ma/gel.The gel was stained using coomassie blue.

were present in the preparation no definitive statements can be made on the sub-unit structure of this enzyme although it is tempting to speculate that the protein is a dimer of apparent molecular weight 70,000 composed of two sub-units of molecular weight 35,000.

The effect of propionate concentration on propionyl CoA synthetase activity is shown in Figure 26. The insets are the Lineweaver-Burk and Eadie-Scatchard plots of the same data. A K_m of 1.28×10^{-3}M was obtained from the Lineweaver-Burk plot (r=.996). A K_m of 1.30×10^{-3}M was obtained from the Eadie-Scatchard plot (r=-.975). Straight line plots were obtained in both cases indicating the presence of only one enzyme with fatty acid activating ability toward propionate. Figure 27 shows the effect of ATP concentration on the activity of propionyl CoA synthetase. A K_m of 9.51×10^{-4}M and 13.33×10^{-4}M for ATP were obtained using the Lineweaver-Burk and Eadie-Scatchard plots of the data (r=.926; r=-.868 respectively). Figure 28 shows the effect of coenzyme A concentration of propionyl CoA synthetase activity. From the Lineweaver-Burk plot a K_m of 5.98×10^{-4}M (r=.997) was obtained. The Eadie-Scatchard plot of the same data gave a K_m of 6.3×10^{-4}M (r=-.964). The effect of various other substrates on enzyme activity is shown in Table 8. The enzyme exhibits maximal activity with propionate followed by acrylate. Some activity is obtained using crotonate and salicyclate as substrates. Adenosine monophosphate is a weak inhibitor of the enzyme (Figure 14).

Figure 26 Effect of propionate concentration on the activity of propionyl CoA synthetase purfied from liver mitochondria.

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M - moles

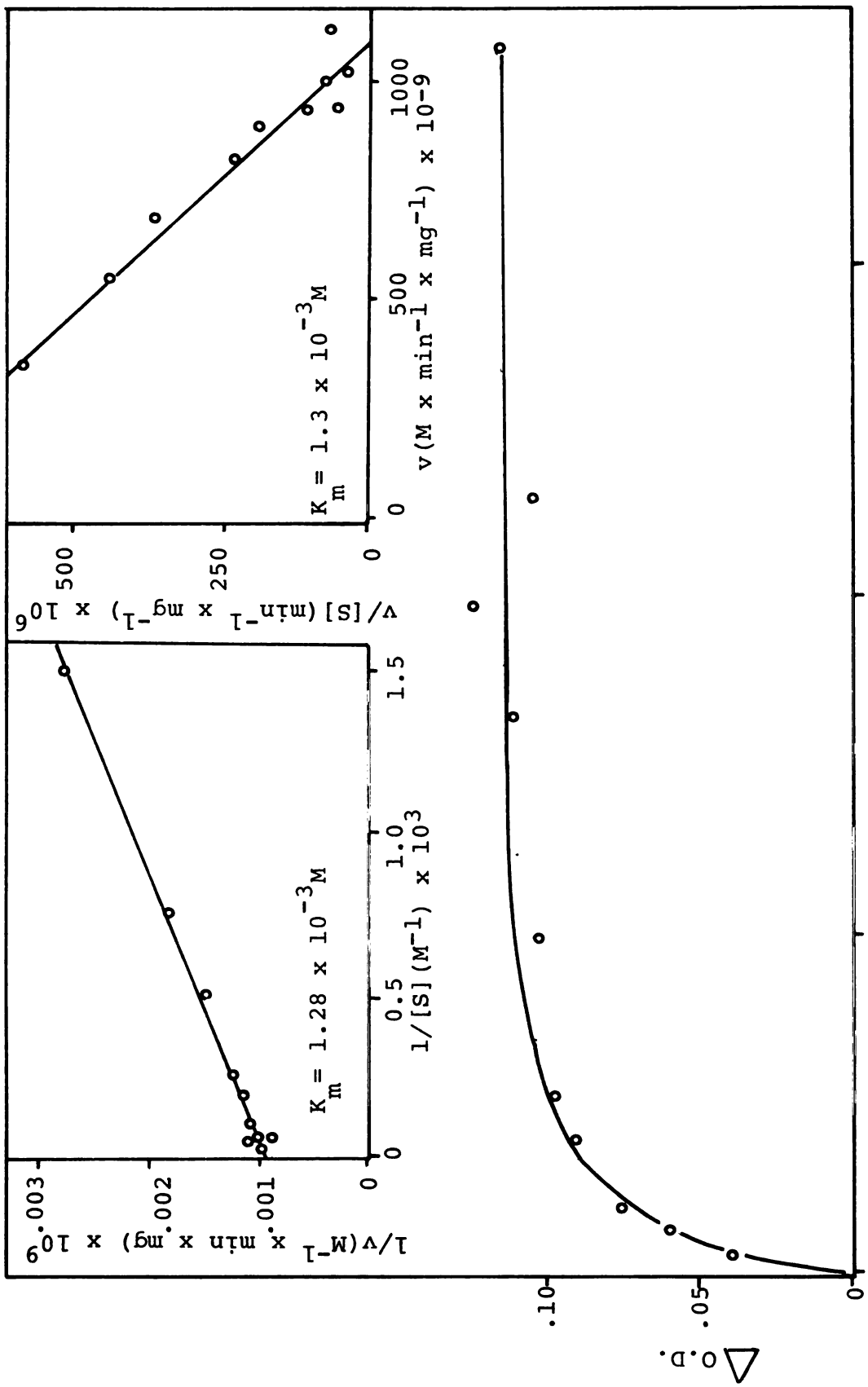

ΔO.D.
.10
.05
0
10
20
30
[S] (M) x 10^-3
v(M x min^-1 x mg^-1) x 10^-9
0
500
1000
K_m = 1.3 x 10^-3 M
v/[S] (min^-1 x mg^-1) x 10^6
0
250
500
1/[S] (M^-1) x 10^3
0.5
1.0
1.5
K_m = 1.28 x 10^-3 M
1/v(M^-1 x min x mg) x 10^9
0
.001
.002
.003

Figure 27 Effect of ATP concentration on the activity of propionyl CoA synthetase purified from liver mitochondria.

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M - moles

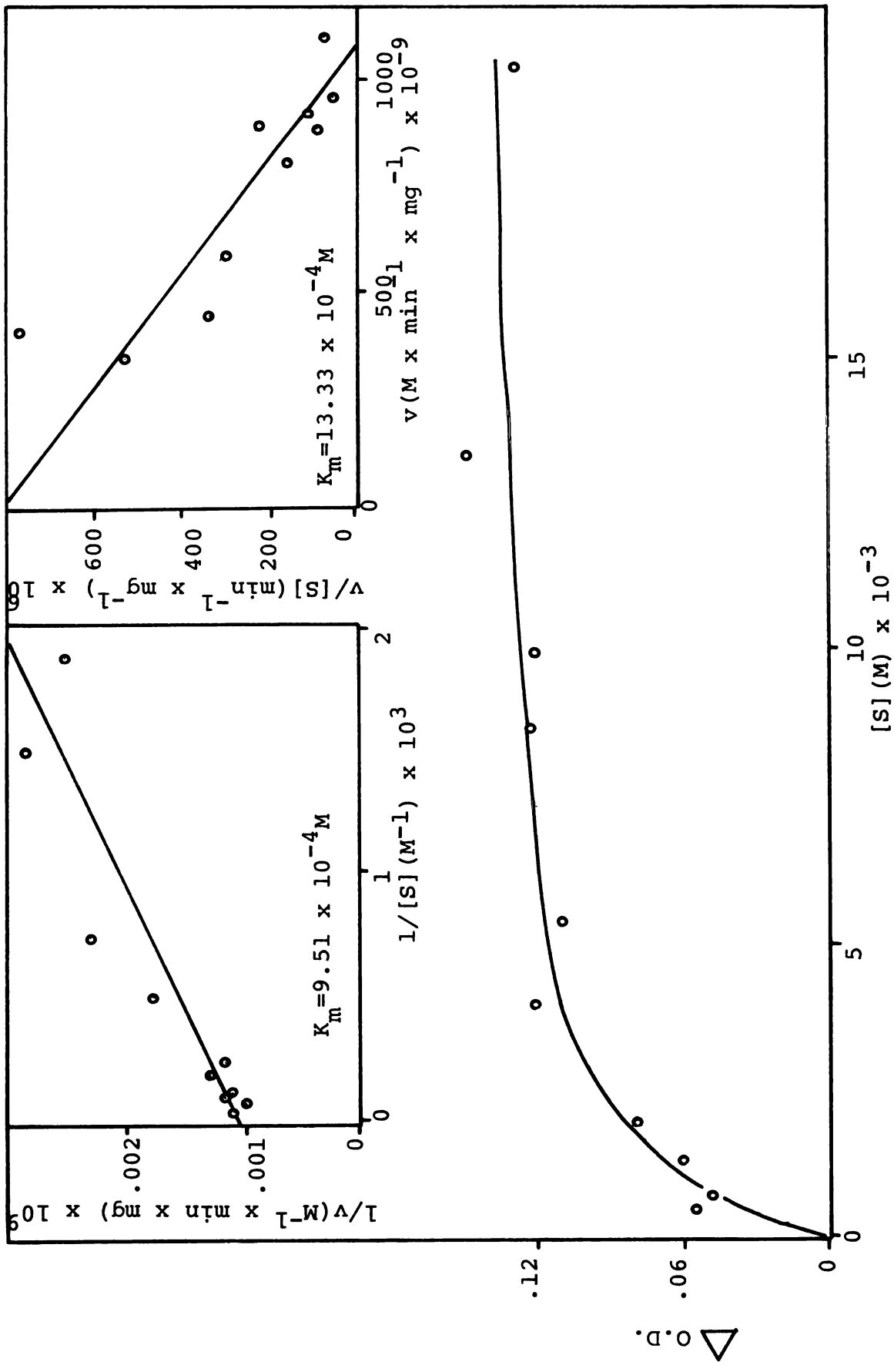

Δ o.D.
.12
.90
0
0
5
10
15
[S](M) x 10^{-3}
v(M x min^{-1} x mg^{-1}) x 10^{-9}
1000_9
500_1
K_m=13.33 x 10^{-4}M
v/[S] (min^{-1} x mg^{-1}) x 10^6
600
400
200
0
1/[S](M^{-1}) x 10^3
1
2
K_m=9.51 x 10^{-4}M
1/v(M^{-1} x min x mg) x 10^9
.002
.001
0

Figure 28 Effect of coenzyme A concentration on the activity of propionyl CoA synthetase purified from liver mitochondria.

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M - moles

..... visual fit of data

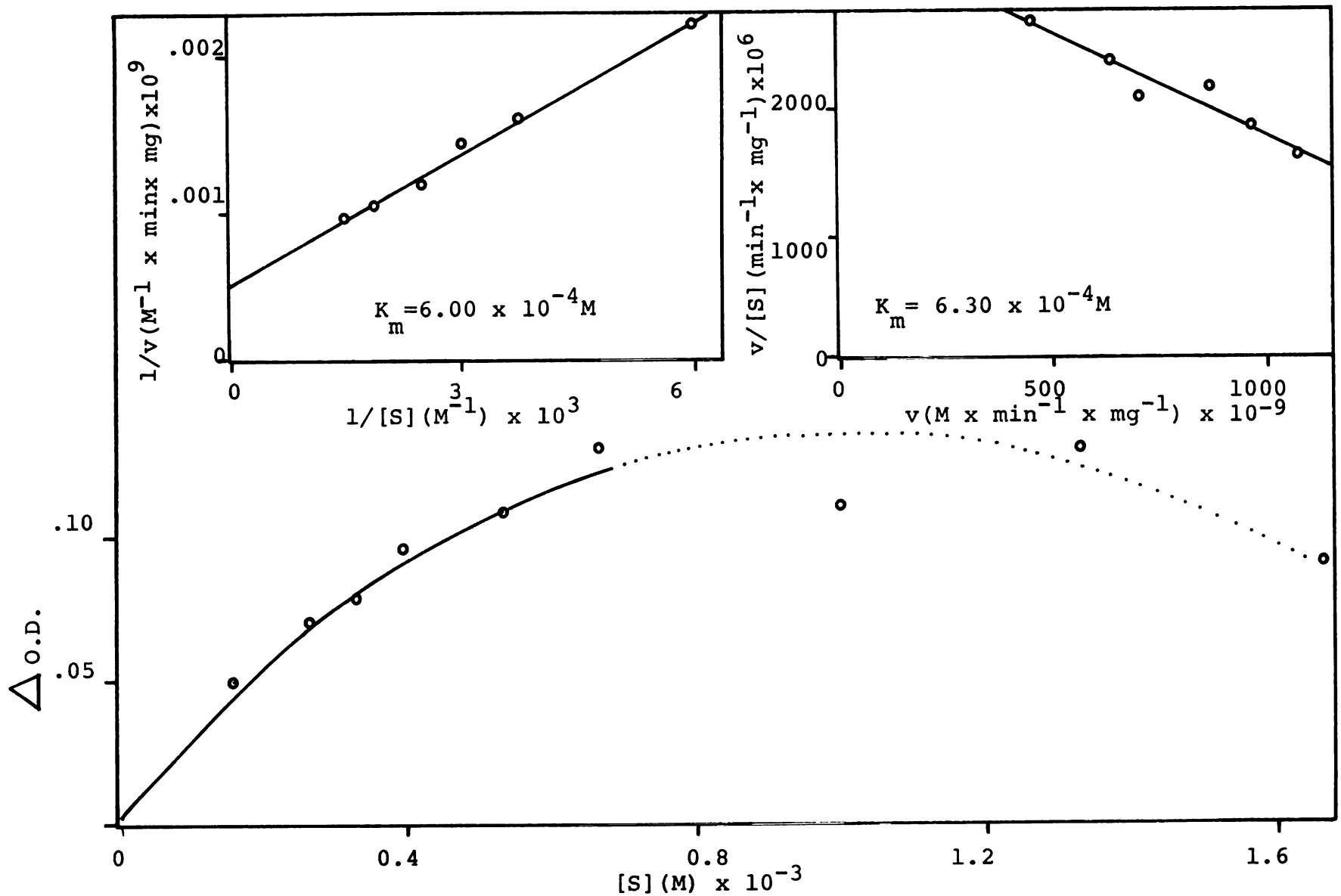

.002
.001
0
1/v(M-1 x minx mg)x10^9
K_m=6.00 x 10^-4M
0
3
6
1/[S](M-1) x 10^3
2000
1000
0
v/[S](min-1x mg-1)x10^6
K_m= 6.30 x 10^-4M
0
500
1000
v(M x min-1 x mg-1) x 10-9
.10
.05
Δ O.D.
0
0.4
0.8
1.2
1.6
[S](M) x 10-3

Table 8 Substrate specificity of propionyl CoA synthetase purified from bovine liver mitochondria.

Substrates Tested	Relative Activity
Acetate	0
Propionate	100
Butyrate	0
Valerate	0
Hexanoate	17
Heptanoate	0
Octanoate	0
Acrylate	85
Maleate	0
Crotonate	13
Fumarate	0
Benzoate	0
Salicyclate	8

The K_i was calculated to be 4.2×10^{-3}M. The enzyme shows a rather broad pH optimum (Figure 13).

The effect of propionate and butyrate concentration on the activity of the butyrate activating enzyme is shown in Figures 29 and 30. In this case Eadie-Scatchard plots of the data did not give straight lines indicating that at least two enzymes with fatty acid activating ability were present. In view of the presence of two enzymes meaningful K_m's cannot be obtained in this case. It is clear however, that this fraction had a low affinity for propionate. In order to obtained OD's of sufficient magnitude for propionate activation, twice the enzyme concentration was used to that employed when butyrate activation was measured at different substrate concentrations (Figures 29 and 30). The possible significance of this observation will be discussed in the next section (discussion). The effect of ATP, coenzyme A and AMP are shown in Figures 31, 32 and 14. As found for the other fatty acid activating enzymes AMP is a weak inhibitor of enzyme acitivity. The effect of pH on enzyme activity using butyrate as a substrate is shown in Figure 13. A pH optimum of 9.0 was obtained. The effect of various substrates on the activity of the butyrate activating enzyme is shown in Table 9. The enzyme had a rather broad affinity for short and medium shain fatty acids. Maximal activity was obtained when benzoate was used as a substrate. Butyrate, valerate, hexanoate and acrylate were all about equally active when used as substrates.

Figure 29 Effect of propionate concentration on the activity of the butyrate activating fraction purfied from liver mitochondria (2x the protein concentration was used in this experiment compared to that used in the experiment of Figure 30).

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M - moles

———— Theoretical line or curve
..... Visual fit of data

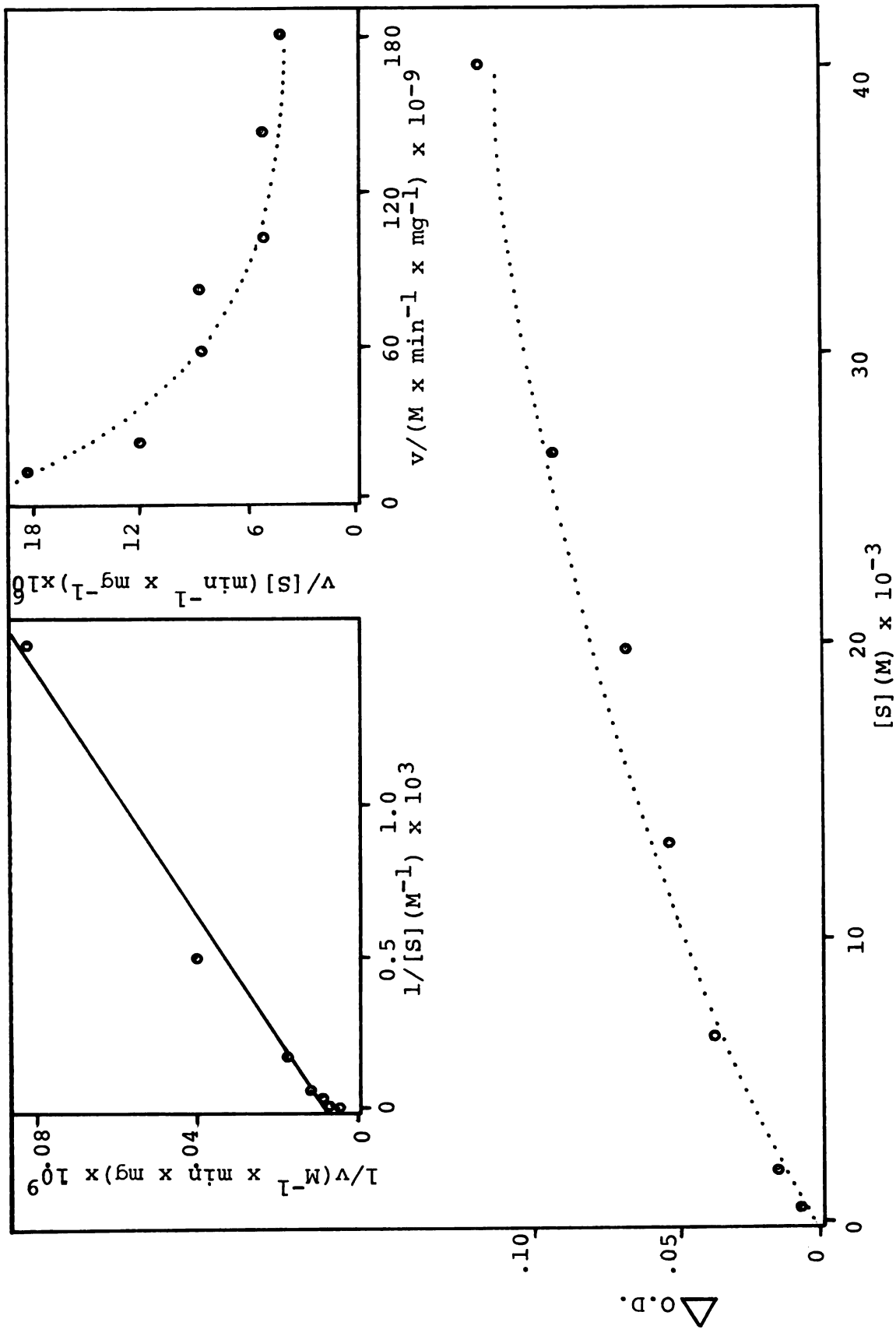

ΔO.D.
0
.05
.10
0
10
20
30
40
[S] (M) x 10^{-3}
$1/v(M^{-1} \times min \times mg) \times 10^9$
0
40
80
0
0.5
1.0
$1/[S]\ (M^{-1})\ \times 10^3$
$v/[S]\ (min^{-1} \times mg^{-1}) \times 10^6$
0
9
12
18
0
60
120
180
$v/(M \times min^{-1} \times mg^{-1}) \times 10^{-9}$

Figure 30 Effect of butyrate concentration on the activity of the butyrate fraction purified from liver mitochondria.

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M- moles

——— Theoretical line or curve
..... Visual fit of data

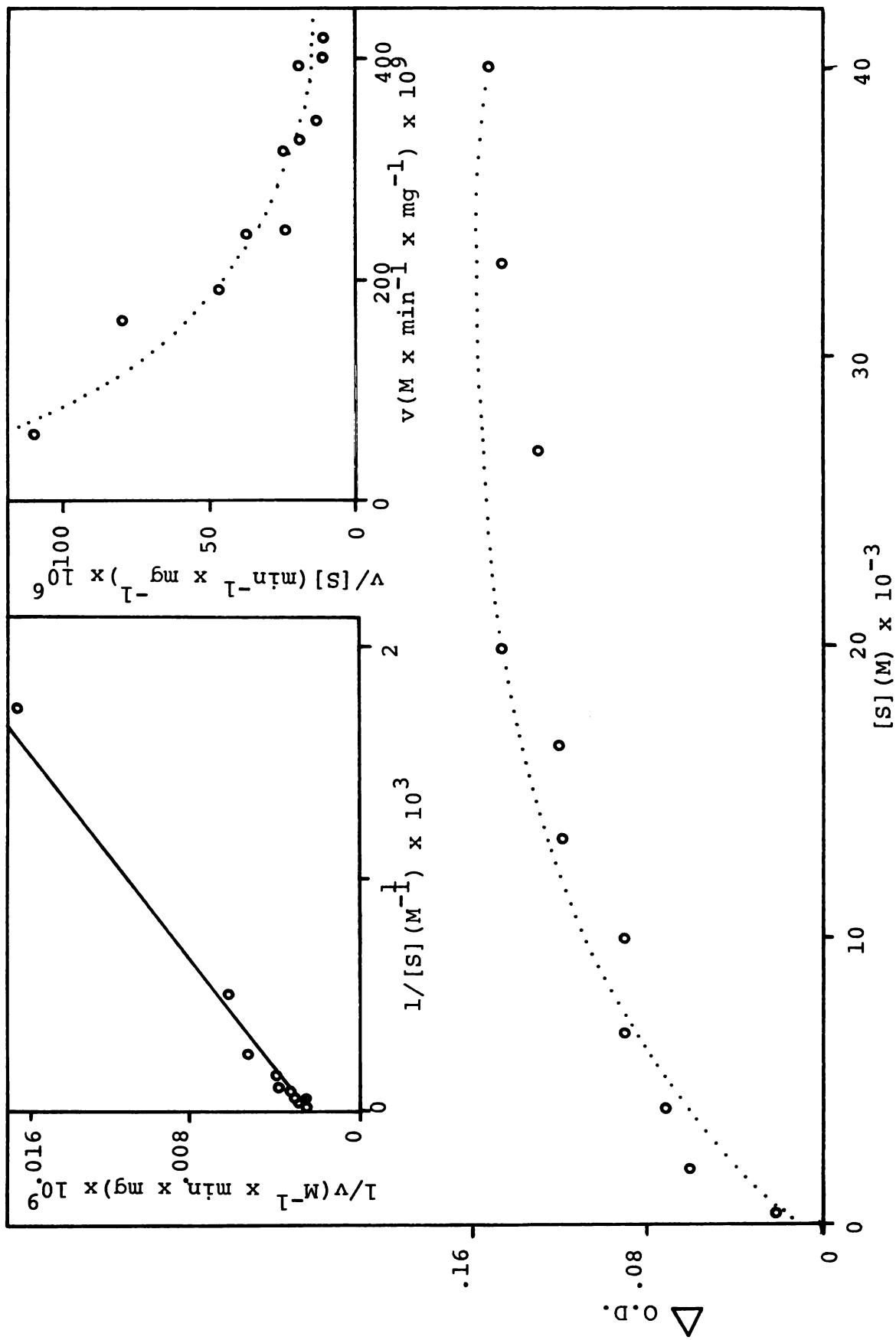

Δ o.D.
.91
.80
0
10
20
30
40
[S] (M) x 10^{-3}
v(M x min^{-1} x mg^{-1}) x 10^9
0
200
400
v/[S] (min^{-1} x mg^{-1}) x 10^6
0
50
100
1/[S] (M^{-1}) x 10^3
0
2
1/v(M^{-1} x min. x mg) x 10^9
0
800
910

Figure 31 Effect of ATP concentration on the activity of the butyrate activating fraction purified from liver mitochondria.

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M - moles

——— Theoretical line or curve
····· Visual fit of data

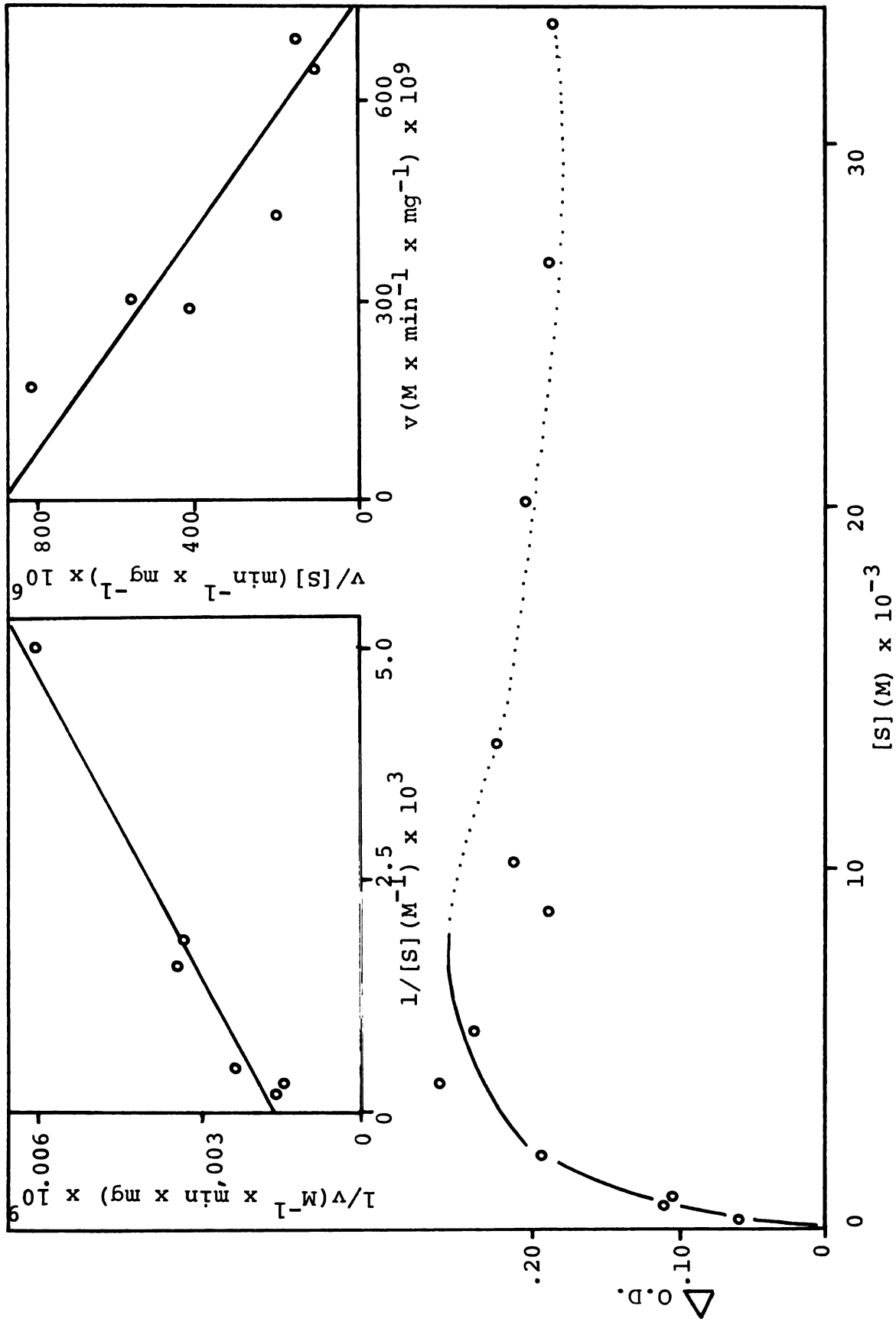

Δ O.D.
0
.10
.20
0
10
20
30
[S] (M) x 10^{-3}
1/v(M^{-1} x min x mg) x 10^9
900
600
300
0
0
2.5
5.0
1/[S] (M^{-1}) x 10^3
v/[S] (min^{-1} x mg^{-1}) x 10^6
800
400
0
0
300
600
v(M x min^{-1} x mg^{-1}) x 10^9

Figure 32 Effect of coenzyme A concentration on the activity of the butyrate activating fraction purified from liver mitochondria.

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M - moles

———— Theoretical line or curve
····· Visual fit of the data

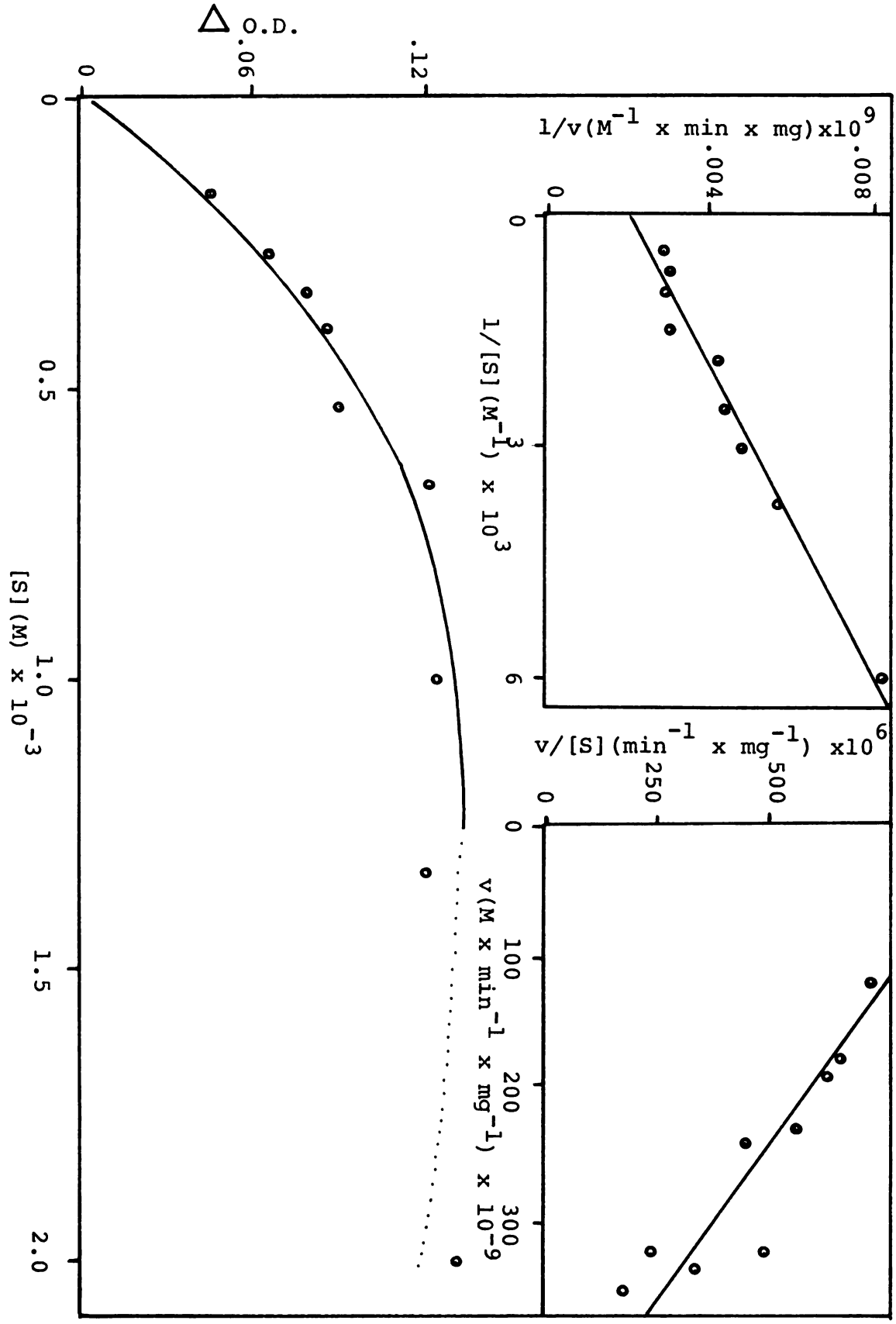

Δ O.D.
0
.06
.12
0
0.5
1.0
1.5
2.0
[S] (M) x 10^-3
1/v(M^-1 x min x mg) x10^9
0
.004
.008
1/[S] (M^-1) x 10^3
0
3
6
v/[S] (min^-1 x mg^-1) x10^6
0
250
500
v (M x min^-1 x mg^-1) x 10^-9
0
100
200
300

Table 9 Substrate specificity of butyrl CoA synthetase purified from bovine liver mitochondria.

Substrates Tested	Relative Activity
Acetate	0
Propionate	32
Butyrate	100
Valerate	127
Hexanoate	111
Heptanoate	67
Octanoate	51
Acrylate	115
Maleate	4
Crotonate	70
Fumarate	24
Benzoate	208
Salicyclate	38

Partial Purification of the Fatty Acid Activating Enzymes from Bovine Kidney Mitochondria

The fatty acid activating enzymes in kidney cortex were isolated by the same methods that had been used to isolate the fatty acid activating enzymes of liver. The mitochondrial extract prepared from kidney tissue activates acetate, propionate, butyrate and valerate (Table 10).

Chromatography of the ammonium sulfate precipitate on DEAE-23 cellulose separated the fatty acid activating ability into two separate components (Figure 33). One component was similar in substrate specificity to the single component isolated by chromatography of liver ammonium sulfate precipitate on DEAE-23 cellulose (Figure 19); that is, it activated propionate, butyrate and valerate but exhibited low activation towards acetate. The other fraction was similar to the component isolated by chromatography of heart ammonium sulfate precipitate on DEAE-23 cellulose (Figure 7); that is it activated acetate and propionate but showed little ability to activate butyrate and valerate. These two fractions isolated from kidney tissue on DEAE-23 cellulose have been designated as the L and H fractions respectively. H is intended to represent the similarity of this fraction to the acetyl CoA synthetase isolated from heart mitochondria whereas L is intended to represent the similarity of the second fraction to the fatty acid activating components of liver mitochondrial tissue.

Table 10 Purification of propionyl CoA synthetase from bovine kidney mitochondria

Weight of tissue used = 2.47 Kg
Wet weight of mitochondria = 174 g

Fraction	Total protein mg	Total units	Specific activity (units/mg protein)[a]			
		C_3	C_2	C_3	C_4	C_5
Mitochondrial suspension	11,375	0	0	0	0	0
Mitochondrial extract[b]	4,160	128,960	31	31	22	14
DEAE-23 cellulose First peak	330	13,860	25	42	60	63
Second peak	570	39,900	100	73	41	31
Calcium phosphate[c] gel First peak	11	3,600	-	327	720	718
Second peak	10	3,246	11	325	30	0

[a] a unit is defined as the amount of enzyme which calalyzes the disappearance of 1 mumole of coenzyme A per minute.

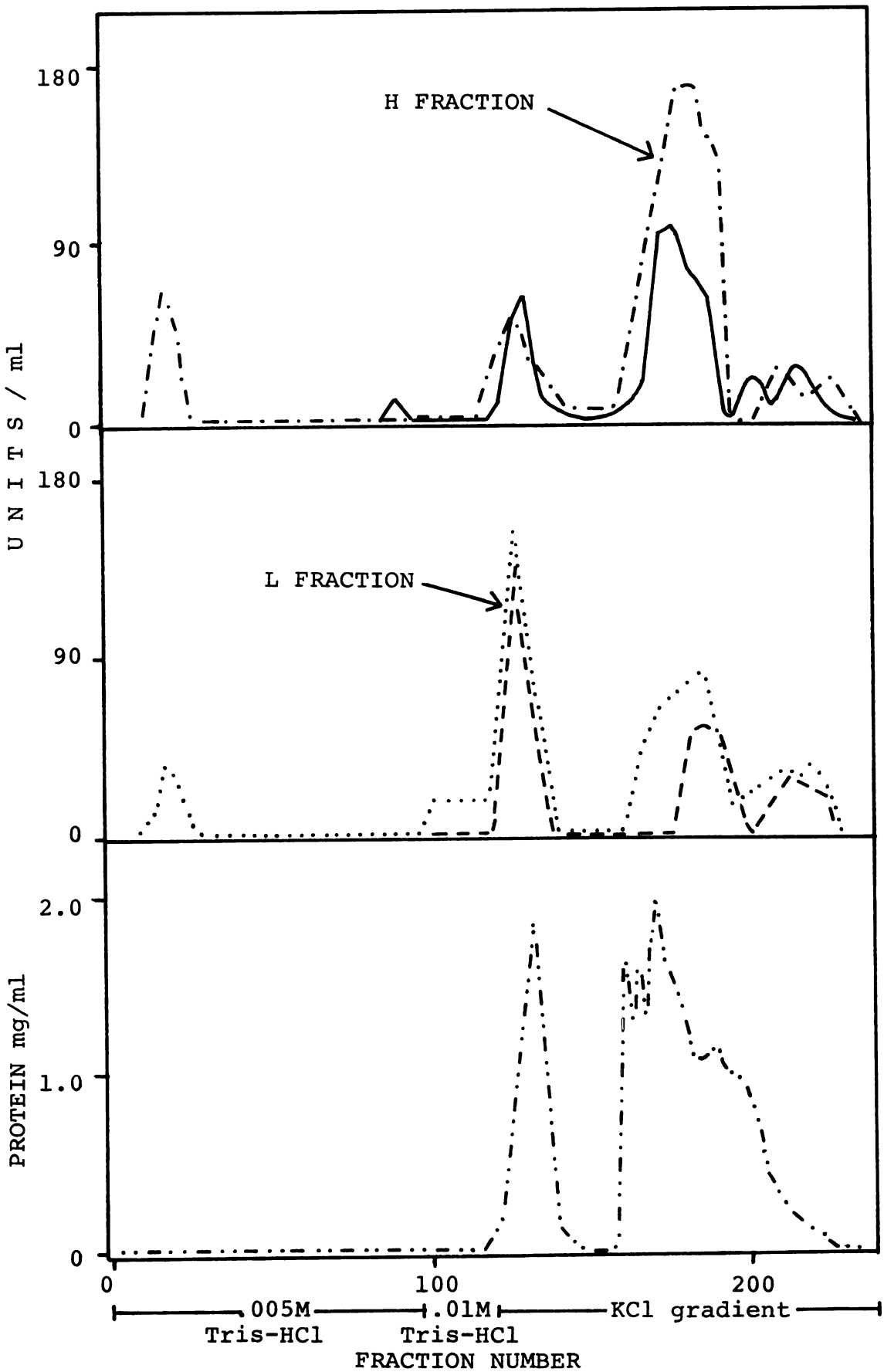
[b] prepared by freezing and thawing the mitochondrial suspension three times.

[c] the first enzyme peak from DEAE-23 cellulose chromatography was used.

Figure 33 Chromatography of the fatty acid activating enzymes of kidney mitochondria on DEAE-23 cellulose.

Column dimensions were 2 cm x 40 cm. Protein was applied to the column. The column was washed with 100 ml of 0.005 M Tris-HCl pH 7.5 followed by 100 ml of 0.01 M Tris-HCl pH 7.5. Enzyme activity was eluted with 600 ml of a linear KCl gradient of 0 to 0.6 M in 0.01 M Tris-HCl ph 7.5. All buffers contained 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was 40 ml/hr. The eluate was collected in 5 ml fractions.

-·-·- acetate activation
——— propionate activation
····· butyrate activation
— — — — valerate activation
-··-·· protein


180
90
0
H FRACTION
U N I T S / ml
180
90
0
L FRACTION
2.0
1.0
0
PROTEIN mg/ml
0
100
200
005M
Tris-HCl
.01M
Tris-HCl
KCl gradient
FRACTION NUMBER

Tubes containing the L fraction were pooled, concentrated using the Dia-flo cell and applied to a calcium phosphate gel column (Figure 34). The L fraction eluted as two separate components with fatty acid activating ability, one component exhibited maximal activity with butyrate and valerate as substrates with lower activity with propionate as a substrate. The other component activated propionate. The L fraction therefore contained two enzymes similar in substrate specificity to the enzymes which had been isolated from liver. Since propionyl CoA synthetase was of primary importance tubes containing this enzyme were pooled and concentrated for further characterization.

In one experiment the H and L fractions obtained from chromatography on DEAE-23 cellulose were combined and concentrated and then applied to a calcium phosphate gel column (Figure 35). Enzyme activity was monitored using only acetate as a substrate. Three components with fatty acid activating ability were isolated. The first eluted with 0.03 M potassium phosphate buffer and probably represents the butyrate activating enzyme, the second eluted with 0.07 M potassium phosphate buffer and represents acetyl CoA synthetase and the last eluted with 0.2 M potassium phosphate buffer and represents propionyl CoA synthetase.

The effect of propionate concentration on the activity of propionyl CoA synthetase is shown in Figure 36. The K_m for propionate is 2.11×10^{-3}M (r=.990) as calculated by the Lineweaver-Burk plot and 2.54×10^{-3}M as calculated by the

Figure 34 Chromatography of the fatty acid activating enzymes of kidney mitochondria on calcium phosphate gel (L fraction prepared from chromatography on DEAE-23 cellulose Figure 33).

Column dimensions were 3 cm x 15 cm. Protein was applied to the column. The column was washed with a stepwise gradient on increasing concentration of potassium phosphate buffer pH 7.0 in 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was 100 ml/hr. The eluate was collected in 5 ml fractions.

——— propionate activation
····· butyrate activation
— — — valerate activation
— ··— ·· protein

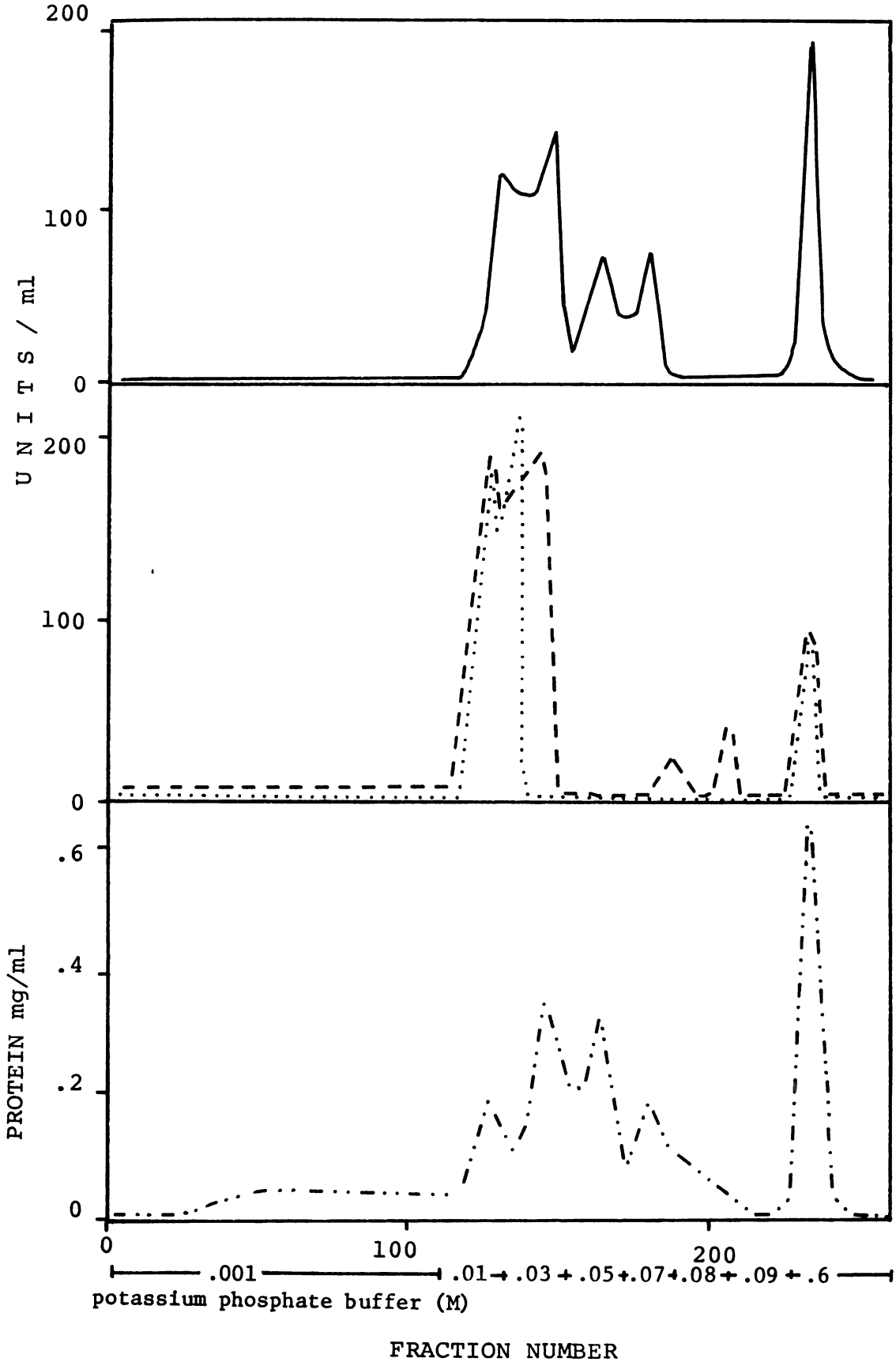

200
100
0
U N I T S / ml
200
100
0
.6
.4
.2
0
PROTEIN mg/ml
0
100
200
.001
.01
.03
.05
.07
.08
.09
.6
potassium phosphate buffer (M)
FRACTION NUMBER

Figure 35 Chromatography of the fatty acid activating enzymes of kidney mitochondria on calcium phosphate gel (H and L fractions prepared from chromatography on DEAE-23 cellulose Figure 33).

Column dimensions were 3 cm x 15 cm. Protein was applied to the column. The column was washed with a stepwise gradient of increasing concentration of potassium phosphate buffer pH 7.0 in 10% glycerol and 2.5 mM 2-mercaptoethanol. Flow rate was 50 ml/hr. The eluate was collected in 5 ml fractions.

-·—·- acetate activation
-··-·· protein

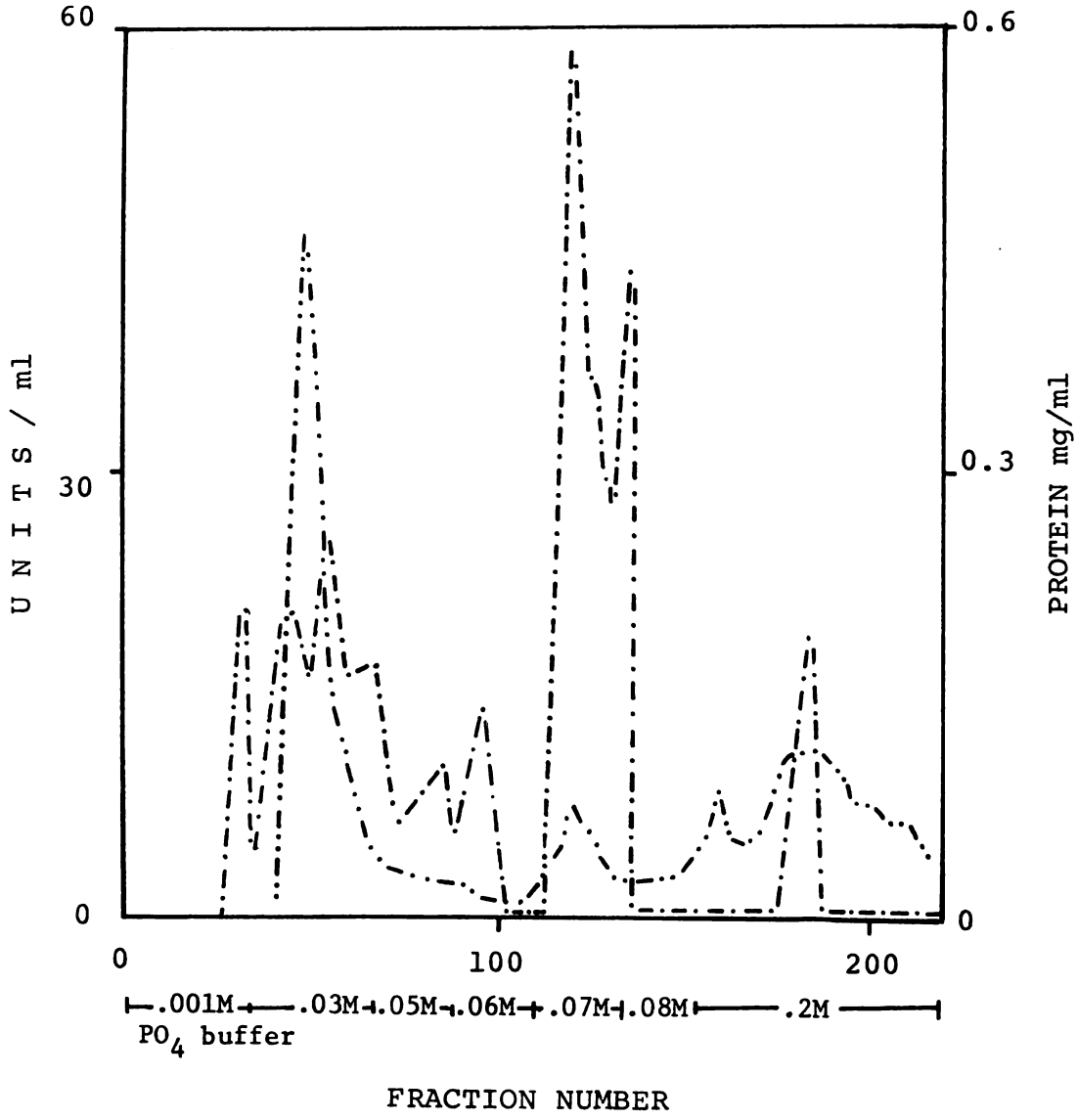
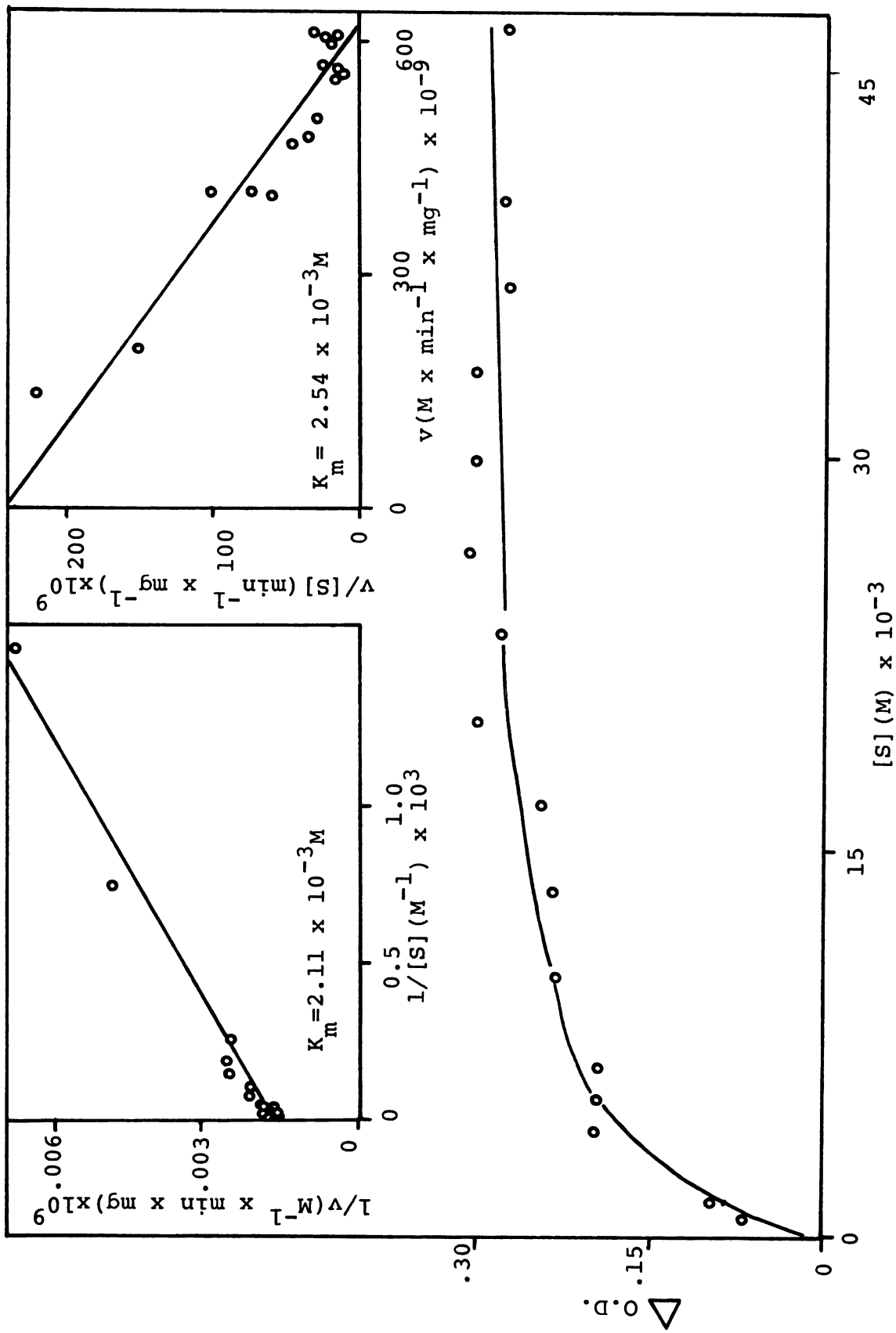

60
30
0
0.6
0.3
0
U N I T S / ml
PROTEIN mg/ml
0
100
200
.001M
.03M
.05M
.06M
.07M
.08M
.2M
PO4 buffer
FRACTION NUMBER

Figure 36 Effect of propionate concentration on the activity of propionyl CoA synthetase purified from kidney mitochondria.

The inset on the left is the Lineweaver-Burk plot, and the inset on the right is the Eadie-Scatchard plot of the same data.

M - moles


Δ O.D.
[S] (M) x 10⁻³
v(M x min⁻¹ x mg⁻¹) x 10⁻⁹
1/v(M⁻¹ x min x mg)x10⁹
1/[S] (M⁻¹) x 10³
K_m=2.11 x 10⁻³M
v/[S] (min⁻¹ x mg⁻¹)x10⁹
K_m = 2.54 x 10⁻³M

Eadie-Scatchard plot (r=-.951). Straight line plots were obtained in both cases indicating that only one enzyme with fatty acid activating ability on propionate was present.

The effect of various substrates on enzyme activity is shown in Table 11. The enzyme exhibits maximal activity using propionate and acrylate as substrates. Lower activation can be demonstrated using crotonate as a substrate. The pattern of fatty acid activation is similar to that obtained with the propionyl CoA synthetase isolated from liver (Table 8).

The chromatography of kidney ammonium sulfate precipitate on 5'-AMP-Sepharose 4B is shown in Figure 37. Two fractions with fatty acid activating ability were obtained similar to those obtained by chromatography of liver ammonium sulfate precipitate on this support. It is not clear where acetyl CoA synthetase eluted on this chromatogram. Loss of acetate units did occur. As described previously when acetyl CoA synthetase of heart tissue was chromatographed on 5'-AMP-Sepharose 4B a similar loss of acetate units occurred.

Hydrophobic chromatography of kidney ammonium sulfate precipitate is shown in Figure 38. Acetate, propionate and butyrate were used as substrates to monitor enzyme activity. The pattern of binding and elution obtained using acetate as a substrate was similar to the pattern obtained for heart tissue (Figure 16). The best separation of enzyme protein activating acetate was that binding to agarose with an alkyl

Table 11 Substrate specificity of propionyl CoA synthetase purified from bovine kidney mitochondria.

Substrates Tested	Relative Activity
Acetate	22
Propionate	100
Butyrate	27
Valerate	0
Hexanoate	3
Heptanoate	0
Octanoate	0
Acrylate	82
Maleate	0
Crotonate	24
Fumarate	14
Benzoate	0
Salicyclate	0

Figure 37 Chromatography of the fatty acid activating enzymes of kidney mitochondria on 5'-AMP-Sepharose 4B.

Column dimensions were 0.6 cm x 20 cm. 30 mg of protein was applied to the column. This was washed on with 36 ml of 0.001 M potassium phosphate buffer pH 7.0. The column was eluted with 24 ml of 0.6 M KCl pH 7.0 followed by 10 ml of 1 M KCl pH 7.0. Flow rate was 15 ml/hr. The eluate was collected in 1.6 ml fractions. All bufferes contained 10% glycerol and 2.5 mM 2-mercaptoethanol.

-·—·- acetate activation
——— propionate activation
····· butyrate activation
—··—·· protein

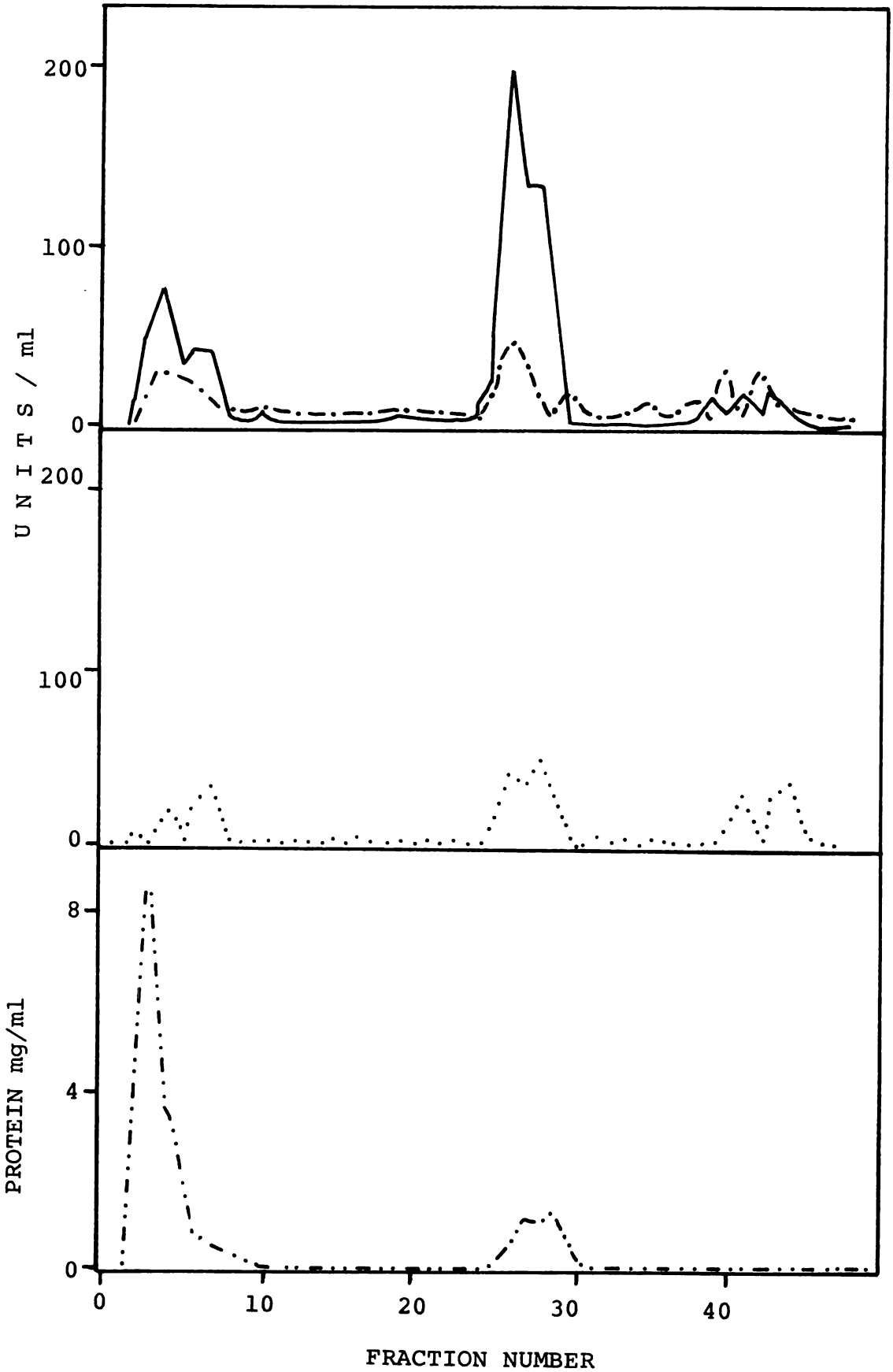

200
100
0
U N I T S / ml
200
100
0
8
4
0
PROTEIN mg/ml
0
10
20
30
40
FRACTION NUMBER

Figure 38 Hydrophobic chromatography of the fatty acid activating enzymes of kidney mitochondria.

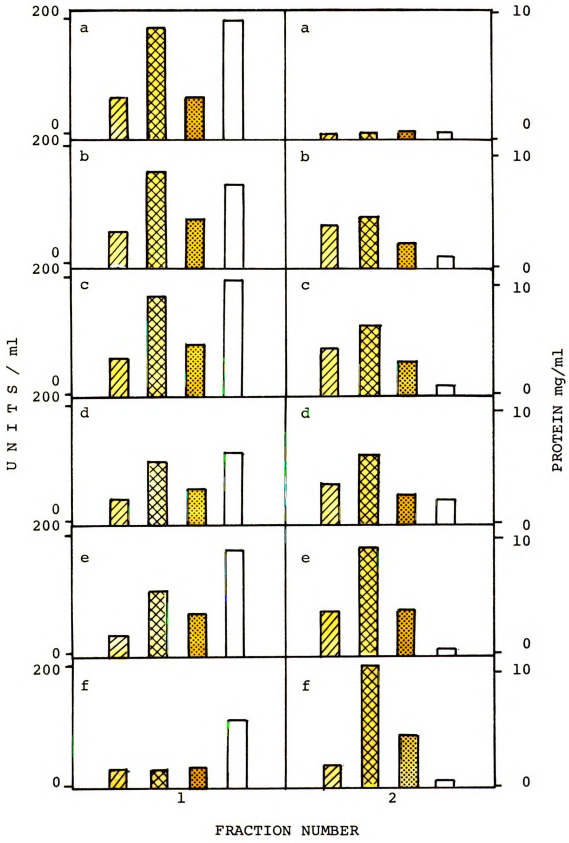
20 mg of protein was placed on each of the six columns (a-f). The protein was washed onto each column with 2 ml of 1 M potassium phosphate buffer pH 7.5. The eluate collected from each column was collected into one tube and designated fraction 1. Each column was then eluted with 2 ml of 0.05 M potassium phosphate buffer pH 7.5 containing 10% glycerol and 2.5 mM 2-mercaptoethanol. The eluate was collected into one tube and designated fraction 2.

a. - Agarose
b. - Agarose - CH_2-CH_3
c. - Agarose -$(CH_2)_3$-CH_3
d. - Agarose -$(CH_2)_5$-CH_3
e. - Agarose -$(CH_2)_7$-CH_3
f. - Agarose -$(CH_2)_9$-CH_3

 acetate activation  protein

 propionate activation

 butyrate activation


200
0
a
b
c
d
e
f
10
0
1
2
U N I T S / ml
PROTEIN mg/ml
FRACTION NUMBER

side chain of four carbon units. A four fold increase in specific activity was obtained. In contrast propionate and butyrate activating protein bound maximally to agarose with alkyl side chain of ten carbon units and could be eluted with 50 mM potassium phosphate buffer. A ten fold increase in specific activity was obtained. The procedure is suitable for separation of the H and L fractions of kidney mitochondria.

Effect of Age and Diet on the Fatty Acid Activating Ability of the Mitochondrial and Cytosolic Fractions of Heart, Kidney and Liver Tissue in the Young Calf

Plasma acetate levels were used as a parameter to estimate substrate availability to the various tissues (Table 12). It was assumed that higher levels would be indicative of an active rumen fermentation process. Acetate concentrations were somewhat variable as evidenced by the rather large standard errors (Table 12). Acetate concentration (mM) was high in the fetus (.627$\pm$.046), fell after birth to .200$\pm$.043 at 14 days of age and then increased with age to .600$\pm$.082 at 60 days of age. The acetate concentration at 14 days of age was significantly lower ($P < .05$) than the value obtained either for the fetus or for animals at 60 days of age. No increase in acetate concentration of plasma was observed between 60 and 120 days of age. The reason for this is not clear since the plasma acetate level in a 2-3 year old Holstein cow is

Table 12 Plasma acetate levels (mM) in the peripheral blood of calves.

AGE (days)	ACETATE (mM)[a]
-14	.627±.046[c]
0	.358±.041[bc]
1	.347±.117[bc]
7	.358±.041[bc]
14	.200±.043[b]
40	.523±.096[bc]
60	.600±.082[c] (.493±.09)
120	.556±.048[bc] (.324±.048)

[a] figures in parentheses are the mean values for animals maintained on a liquid diet

[bc] means sharing a common superscript are not significantly different $P < .05$

2-3 mM (Ricks, 1978) and therefore plasma acetate levels would be expected to be increasing as the animal matures.

Blood acetate values for animals maintained on a liquid diet for 120 days were lower than the values obtained for comparable animals maintained on solid feed (Table 12). The difference approached statistical significance ($P < .1$). However, at 60 days of age blood acetate levels were not different between the two groups. The reason for this anomaly is not clear. At slaughter the rumen of all calves was observed for papillary development. Calves fed the liquid diet showed the typical lack of papillary development whereas animals fed solid feed had well developed papillae characteristic of an active rumen fermentation process. Three of the five calves fed the liquid diet had plasma acetate values in excess of 0.5 mM whereas the other two calves had plasma acetate values of .25 mM. Since the papillary development in all these calves was negligible it is possible that in the three calves with high plasma acetate levels a substantial production of endogenous acetate was occurring for some unknown reason.

No significant correlations ($P < .05$) of blood acetate to the fatty acid activating ability of the various tissues could be found. However, when correlations were determined using the means for animals within each age group significant correlations ($P < .05$) were obtained. The variabiltiy of the blood acetate values within each group probably accounts for this. These correlations must therefore be interpreted

with caution. However, it is believed that where significant effects of diet and significant correlations of blood acetate with enzyme activity were obtained then this was strong evidence for a role of rumen fermentation, indirectly or directly, in influencing the ability of a organ or tissue to activate volatile fatty acids.

Acetate and propionate activation in the mitochondria and cytosol fractions of heart tissue was high in the fetus and at birth (Tables 13 and 14), declined after birth and then increased progressively as the animal matured. Enzyme activity was significantly greater both in the fetus and at 120 days of age ($P < .05$) to values obtained at 14 and 40 days of age (Table 13). In both the mitochondrial and cytosolic fraction butyrate and valerate activation were low relative to acetate and propionate activation and showed less tendency to increase with age (Tables 13 and 14). No effect of diet on the acetate and propionate activation of mitochondria could be detected. However, the cytosolic activation of these substrates was influenced by diet ($P < .05$). There was no significant diet by age interaction in this case.

Plasma acetate concentration and acetate activating ability of the mitochondrial fractions of heart tissue were not correlated. However, a significant correlation was obtained ($r=.8348$, $P < .01$) for acetate concentration with cytosolic acetate activating ability if the values for the fetus and newborn animal were omitted.

Table 13 Effect of age and diet on the specific activity (units/mg protein) of the fatty acid activating enzymes of heart mitochondria.

Substrate tested	DAYS OF AGE -14	0	1	7	14	40	60		120	
Acetate	20 ± 5^{bc}	18 ± 4^{bc}	23 ± 7^{bc}	15 ± 4^{bc}	9 ± 4^{b}	11 ± 1^{b}	23 ± 2^{bc}	(26±4)	33 ± 8^{c}	(36±4)
Propionate	14 ± 2^{b}	14 ± 4^{b}	15 ± 7^{b}	16 ± 1^{b}	7 ± 4^{b}	6 ± 1^{b}	13 ± 1^{b}	(17±3)	20 ± 4^{b}	(17±4)
Butyrate	8 ± 2^{bc}	8 ± 1^{bc}	12 ± 5^{bc}	12 ± 3^{b}	1 ± 1^{c}	1 ± 1^{c}	1 ± 1^{c}	(5±2)	10 ± 5^{b}	(3±1)
Valerate	5 ± 2^{bc}	8 ± 3^{bc}	13 ± 4^{b}	11 ± 1^{bc}	3 ± 1^{bc}	2 ± 1^{bc}	1 ± 1^{c}	(4±2)	10 ± 5^{bc}	(5±2)
n	5	4	3	4	4	5	5	(5)	3	(8)

a figures in parentheses are the mean values ± the standard error for animals maintained on a liquid diet.

bcd means sharing a common superscript are not significantly different $P < .05$

Table 14 Effect of age and diet on the specific activity (units/mg protein) of the fatty acid activating enzymes of the cytosolic fraction of heart tissue.

Substrate tested	DAYS OF AGE -14	0	1	7	14	40	60[a]	120[a]
Acetate	6 ± 2^{b}	17 ± 2^{bc}	7 ± 2^{b}	8 ± 1^{b}	6 ± 3^{b}	14 ± 2^{b}	$30\pm4^{d}(24\pm3)^{**}$	$28\pm7^{cd}(16\pm5)^{**}$
Propionate	7 ± 2^{b}	15 ± 3^{b}	9 ± 1^{b}	9 ± 3^{b}	12 ± 3^{b}	14 ± 2^{b}	$20\pm3^{b}(16\pm3)^{**}$	21 ± 6^{b} $(10\pm4)^{**}$
Butyrate	4 ± 1^{b}	11 ± 3^{b}	6 ± 1^{b}	7 ± 3^{b}	6 ± 2^{b}	9 ± 1^{b}	5 ± 1^{b}(4 ± 1)	7 ± 1^{b} (5 ± 2)
Valerate	5 ± 2^{b}	11 ± 2^{b}	7 ± 2^{b}	8 ± 2^{b}	7 ± 1^{b}	7 ± 1^{b}	5 ± 1^{b}(7 ± 2)	9 ± 2^{b} (4 ± 1)
n	5	4	4	4	4	5	5 (5)	3 (8)

[a] figures in parentheses are the mean values $\pm$ the standard error for animals maintained on a liquid diet. These are significantly different from the means of animals maintained on a normal diet for the same number of days at ** $P < .05$

[bcd] means sharing a common superscript are not significantly different $P < .05$

The fatty acid activating ability of kidney mitochondria is low at birth and increases with age (Table 15). For nearly every substrate the fatty acid activation at 120 days of age was significantly greater ($P < .05$) than that in the fetus or in animals at 0,1,7,14 and 40 days of age. A similar pattern was observed for the cytosolic fraction of kidney (Table 16).

Although there was no detectable effect of diet on the cytosolic activation of kidney tissue a significant effect of diet was found for the mitochondrial fraction. In each case a significant diet by age interaction occurred. Hence the main effects were tested separately within each age group. At both 60 and 120 days of age animals fed a liquid diet had significantly lower propionate activation ($P < .05$, $P < .001$ respectively) than animals fed solid feed (Table 15). Butyrate and valerate activation (Table 15) at 120 days of age were also significantly lower ($P < .001$, $P < .001$ respectively) than the corresponding activation obtained for animals fed solid feed. In addition significant correlations for mitochondrial propionate ($r=.7356$, $P < .02$) and for butyrate activation ($r=.6996$, $P < .05$) with blood acetate over all ages and all diets were obtained (Table 19). Acetate activation was lower at 120 days of age for the liquid fed group. The difference approached statistical significance ($P < .1$).

As expected acetate activation by either mitochondrial or cytosolic fractions of liver tissue was low in the fetus

Table 15 Effect of age and diet on the specific activity (units/mg protein) of the fatty acid activating enzymes of kidney mitochondria.

Substrate tested	DAYS OF AGE -14	0	1	7	14	40	60[a]	120[a]
Acetate	2±1[b]	2±1[b]	5±2[b]	3±1[b]	3±1[b]	7±3[b]	12±4[bc] (9±1)	20±6[c] (12±1)*
Propionate	6±2[bc]	3±1[b]	4±2[bc]	6±4[bc]	2±1[b]	10±3[bc]	16±1[cd] (8±2)**	29±3[d] (9±1)****
Butyrate	3±1[b]	2±1[b]	3±1[b]	4±4[b]	ND[b]	3±1[b]	8±2[bc] (5±1)	17±5[c] (3±0.3)****
Valerate	3±2[b]	2±0.5[b]	3±0.5[b]	3±2[b]	1±0.4[b]	3±1[b]	6±2[b] (4±1)	15±2[c] (3±0.4)****
n	5	4	3	4	4	5	5 (5)	3 (8)

[a] figures in parentheses are the mean values ± standard error for animals maintained on a liquid diet. These are significantly different from the means of animals maintained on a normal diet for the same number of days at * $P < .1$, ** $P < .05$, *** $P < .005$, **** $P < .001$

[bcd] means sharing a common superscript are not significantly different $P < .05$

Table 16 Effect of age and diet on the specific activity (units/mg protein) of the fatty acid activating enzymes of the cytosolic fraction of kidney tissue.

Substrate tested	DAYS OF AGE -14	0	1	7	14	40	60[a]	120[a]
Acetate	1±1[b]	3±1[b]	3±1[b]	6±1[bc]	5±1[bc]	7±1[bc]	10±2[c] (9±1)	9±1[bc] (12±1)
Propionate	6±2[b]	8±3[bc]	6±2[b]	12±2[bc]	11±3[bc]	17±2[cd]	24±3[d] (25±2)	27±1[d] (29±1)
Butyrate	3±1[b]	4±2[b]	4±1[b]	6±1[b]	5±2[b]	9±1[b]	8±2[b] (10±1)	12±1[b] (10±1)
Valerate	3±2[b]	4±1[b]	4±1[b]	5±1[b]	4±2[b]	7±1[b]	7±2[b] (7±1)	10±1[b] (8±2)
n	5	4	4	4	4	5	5 (5)	3 (8)

[a] figures in parentheses are the mean values ± the standard error for animals maintained on a liquid diet.

[bcd] means sharing a common superscript are not significantly different $P < .05$

and at birth and did not increase with age (Tables 17 and 18) confirming that liver tissue contains negligible quantities of acetyl CoA synthetase at all times. Propionate, butyrate, and valerate activation was also low in the fetus and at birth in mitochondrial and cytosolic fractions however a significant effect of age was obtained. Propionate activation in the liver mitochondria was significantly higher at 120 days of age ($P < .05$) than values obtained for the fetus and for animals at birth and at 1, 14 and 40 days of age (Table 17). A similar pattern was observed when butyrate was used as the substrate to measure enzyme activity (Table 17). The cytosolic activation of propionate, butyrate, and valerate also tended to increase with age but this effect was not as pronounced as the mitochondrial increase (Table 18).

A statistically significant effect of diet at the $P < .05$ level was not obtained for propionate, butyrate, and valerate activation of liver mitochondrial or cytosolic fractions. However, the data do suggest that animals maintained on a liquid diet do have lower fatty acid activation in the mitochondria which approaches statistical significance ($P < .2$). For the mitochondria, propionate activation (mμmoles/min/mg protein) at 120 days of age was 27 ± 5 and 15 ± 4 for animals maintained on a normal and liquid diet respectively (Table 17). At 60 days of age the corresponding (Table 17) were 25 ± 5 and 20 ± 7 respectively. Moreover, there was a positive correlation between plasma acetate

Table 17 Effect of age and diet on the specific activity (units/mg protein) of the fatty acid activating enzymes of liver mitochondria.

Substrate tested	DAYS OF AGE -14	0	1	7	14	40	60[a]	120[a]
Acetate	2 ± 0.75^{b}	2 ± 1^{b}	1 ± 1^{b}	3 ± 2^{b}	1 ± 1^{b}	1 ± 1^{b}	5 ± 1^{b} (3 ± 1)**	4 ± 2^{b} (2 ± 1)**
Propionate	6 ± 2^{b}	8 ± 2^{b}	3 ± 2^{b}	10 ± 3^{bcd}	5 ± 1^{b}	9 ± 2^{bc}	25 ± 5^{cd} (20±7)	27 ± 5^{d} (15±4)
Butyrate	3 ± 1^{b}	6 ± 1^{bc}	2 ± 1^{b}	6 ± 1^{bc}	3 ± 1^{b}	8 ± 1^{bcd}	13 ± 2^{cd} (11±4)	17 ± 4^{d} (9±3)
Valerate	3 ± 2^{b}	5 ± 1^{b}	1 ± 0.3^{b}	6 ± 1^{b}	4 ± 1^{b}	8 ± 2^{b}	10 ± 1^{b} (11±4)	9 ± 4^{b} (8±2)
n	5	4	3	4	4	5	5 (5)	3 (8)

[a] figures in parentheses are the mean values ± standard error for animals maintained on a liquid diet. These are significantly different from the means of animals maintained on a normal diet for the same number of days at ** $P < .05$

[bcd] means sharing a common superscript are not significantly different $P < .05$

Table 18 Effect of age and diet on the specific activity (units/mg protein) of the fatty acid activating enzymes of the cytosolic fraction of liver tissue.

Substrate tested	DAYS OF AGE -14	0	1	7	14	40	60[a]	120[a]
Acetate	2 ± 1^{b}	3 ± 2^{b}	1 ± 1^{b}	2 ± 1^{b}	4 ± 1^{b}	6 ± 1^{b}	10 ± 5^{b} (2 ± 0.5)**	7 ± 1^{b} (4 ± 1)**
Propionate	5 ± 1^{b}	12 ± 4^{bc}	7 ± 2^{b}	10 ± 2^{bc}	14 ± 2^{bc}	22 ± 3^{c}	17 ± 2^{bc}(14 ± 1)*	21 ± 3^{bc}(14 ± 2)*
Butyrate	2 ± 1^{b}	5 ± 2^{bc}	3 ± 1^{b}	5 ± 1^{b}	7 ± 2^{bc}	13 ± 2^{c}	10 ± 2^{bc}(7 ± 0.5)	10 ± 2^{bc}(9 ± 1)
Valerate	3 ± 1^{b}	5 ± 2^{bc}	3 ± 1^{b}	3 ± 0.5^{b}	7 ± 2^{bc}	11 ± 1^{c}	7 ± 2^{bc}(6 ± 0.5)	9 ± 2^{bc}(9 ± 1)
n	5	4	3	4	4	5	5 (5)	3 (8)

[a] figures in parentheses are the mean values ± standard error for animals maintained on a liquid diet. These are significantly different from the means of animals maintained on a normal diet for the same number of days at * $P < .1$, ** $P < .05$

[bcd] means sharing a common superscript are not significantly different $P < .05$

concentrations and propionate, butyrate, and valerate activation by liver mitochondria ($r=.7650$, $P < .02$, $r=.8034$, $P < .01$, $r=.7177$, $P < .05$ respectively).

A significant effect of diet ($P < .05$) on acetate activation by liver mitochondrial and cytosol fractions was obtained (Table 17 and 18) although there were no significant correlations of enzyme activity with blood acetate in this case (Tables 19 and 20). In view of the low levels of acetyl CoA synthetase in liver it is doubtful whether this observation is of any physiological significance and thus will not be discussed further.

Table 19 Correlation coefficients of fatty acid activating ability in the mitochondrial fractions of heart, kidney and liver tissue with peripheral blood acetate concentration.

ORGAN		SUBSTRATE TESTED			
		acetate	propionate	butyrate	valerate
HEART	r	.2405	.1998	-.0167	-.0986
	n	10	10	10	10
	S	NS	NS	NS	NS
KIDNEY	r	.6436	.7356	.6966	.6327
	n	9	9	9	9
	S	NS	.02	.05	NS
LIVER	r	.6847	.7650	.8034	.7177
	n	9	9	9	9
	S	NS	.02	.01	.05

Table 20 Correlation coefficients of fatty acid activating ability in the cytosolic fractions of heart, kidney and liver tissue with peripheral blood acetate concentration.

ORGAN		SUBSTRATE TESTED			
		acetate	propionate	butyrate	valerate
HEART*	r	.8438	.7998	.0986	.0900
	n	8	8	8	8
	S	.01	.02	NS	NS
KIDNEY	r	.4624	.5496	.7740	.6257
	n	9	9	9	9
	S	NS	NS	NS	NS
LIVER	r	.6508	.6313	.5889	.3747
	n	9	9	9	9
	S	NS	NS	NS	NS

* -14 and 0 day values omitted

DISCUSSION

The results clearly demonstrate that the differences in short chain fatty acid activating ability of mitochondrial extracts of heart, kidney, and liver are due to the presence of different enzymes with overlapping substrate specificities. Moreover, the presence of these different enzymes in the different tissues can be related to the physiological function of that tissue.

Volatile Fatty Acid Activating Enzymes of Heart Tissue

By using procedures which ensured that the unstable enzymes activating propionate, butyrate, or valerate were stabilized and by monitoring all column effluents using acetate, propionate, butyrate, and valerate it was established that heart mitochondrial tissue contains predominantly one enzyme, acetyl CoA synthetase, with short chain fatty acid activating properties. The enzyme activates primarily acetate and propionate (Figures 7 and 8, Tables 5 and 6). Some evidence for an enzyme which activates butyrate was obtained (Table 5, Figure 7) and this probably is the same enzyme purified by Webster *et al*. (1965) from bovine heart mitochondria. However, the activity of this enzyme relative to that of acetyl CoA synthetase is very low.

The physiological function of acetyl CoA synthetase in heart tissue would be to oxidize acetate, obtained from peripheral blood, to generate ATP needed for maintaining physiological function i.e. pumping of blood. Heart tissue can also use long chain fatty acids as a source of energy and thus the physiological function of the butyrate enzyme (Webster et al. 1965) would possible be the β-oxidation of medium chain fatty acids for energy. It is unlikely that the enzyme takes up ruminally derived butryate since butyrate is mainly metabolized by the rumen epithelial tissue to β-hydroxybutyrate. Little butyrate appears in portal or peripheral blood. Thus, only the acetate activating enzyme is of any significance in terms of the animals ability to use ruminally derived volatile fatty acids.

Acetyl CoA synthetase was judged to be pure by the technique of polyacrylamide gel electrophoresis. Electrophoresis in the presence of SDS gave one band indicating that the enzyme was probably composed of a single polypeptide chain. The purified enzyme showed a tendency to bind to glass. This is a property which was not shared by the acyl CoA synthetases of liver. Moreover, it was a stable enzyme relative to the other acyl CoA synthetases. Both these properties may be related to the fact that the enzyme is a glycoprotein (Figure 9, Table 4). Enzymes which are glycoproteins are relatively stable to degradation by proteolytic enzymes (Coffey and DeDuve, 1968) and are

remarkably stable on storage and at elevated temperatures (Razur et al., 1970; Arnold, 1069). In addition enzymes composed of a single polypeptide chain are generally more stable than oligomeric enzymes (Segal, 1976).

Acetyl CoA synthetase has been purified from bovine mammary gland mitochondria and this enzyme has many properties which are similar to those of the acetyl CoA synthetase isolated from heart tissue. Both are glycoproteins, both readily aggregate, have similar apparent molecular weights and behave similarly on chromatography on DEAE-23 cellulose and calcium phosphate gel. The affinity of the enzyme for acetate is similar. The K_m is 6.1×10^{-4}M for the mammary acetyl CoA synthetase (Qureshi, 1971) and the K_m for the heart acetyl CoA synthetase is 1.79×10^{-4}M (Figure 11). The mammary enzyme has a similar pattern of substrate activation as the heart enzyme (Table 6). Both enzymes have a high affinity for acrylate. The relative activity of heart acetyl CoA synthetase for acetate, propionate and acrylate is 100:67:141 (Table 6) whereas the relative activity of the mammary gland enzyme for these substrates is 100:65:141 (Qureshi, 1971). Both enzymes are active over a rather broad pH range and both are weakly inhibited by AMP (Figure 13, Qureshi, 1971).

The enzymes are however, probably not identical. The mammary enzyme contains fucose, galactose, glucose, and N-acetyl-neuraminc acid (Stamoudis, 1974) whereas the heart enzyme contains mannose, galactose, glucose,

N-acetyl-galactosamine, and N-acetyl-glucosamine, but no fucose (Table 4). The activity of the enzyme in mammary tissue is dependent on the stage of lactation (Marinez et al., 1976) whereas the enzyme in heart tissue probably does not demonstrate large fluctuations in activity as a result of changes in physiological state (Ricks, 1978).

The acetate concentration in the peripheral blood of the dairy cow is approximately 1-3 mM (Ricks, 1978). Acetate concentration can increase slightly after feeding (Ross and Kitts, 1973). The K_m for acetate is 1.79×10^{-4}M and 6.1×10^{-4}M for the heart and mammary acetyl CoA synthetases respectively. Thus the heart enzyme will be working at half maximal velocity when the acetate concentration is approximately 0.179 mM. Acetate concentration in peripheral blood is far greater than this and since acetate diffuses freely across the cytoplasm of the cell, acetate concentration per se probably does not control its rate of uptake by heart or lactating mammary gland mitochondria. The enzyme will be saturated with substrate at all physiological concentrations of acetate. Presumably both heart and lactating mammary tissue are critically dependent on a constant supply of acetate for energy. In the former case it is required for mechanical work i.e. the pumping of blood and in the latter case for the synthesis and secretion of milk. Thus the kinetic properties of the enzyme ensure that as much acetate as is available will be taken up and that fluctuations in substrate (acetate) availability

will not influence enzyme activity. Although in this work the kinetic properties of the acetyl CoA synthetase of kidney were not studied it seems reasonable to suppose that its K_m for acetate would be in the same range as that of heart and mammary tissue, that the kidney also utilizes acetate as an energy furnishing substrate necessary for its physiological function and that large amounts of acetate will always be taken up irrespective of small fluctuations in plasma acetate levels.

Uptake of acetate by peripheral tissues in the adult ruminant is therefore not controlled simply by substrate availability. At all physiological concentrations of blood acetate acetyl CoA synthetase will be working at maximal velocity. This does not however, preclude some other mechanism such as feedback inhibition (allosteric regulation) from controlling acetyl CoA synthetase activity *in vivo*. However, this seems unlikely, at least for the heart enzyme, since this enzyme is composed of a single polypeptide chain (Figures 9 and 10) of molecular weight 67,500. In general, allosteric enzymes are composed of a number of polypeptide chains or sub-units. Metabolic regulation of acetate uptake must therefore be governed by the amount of enzyme within a given tissue. It has been established (Marinez *et al.*, 1976) that acetyl CoA synthetase activity in mammary tissue prior to parturition is absent but that activity increases as the gland becomes functional in milk synthesis and secretion. This increase

is due to an increase in enzyme synthesis rather than conversion of an inactive to an active form of the enzyme. Moreover, it appears to be under hormonal regulation. In the adult ruminant acetyl CoA synthetase of heart and kidney tissue does not show these fluctuations with physiological state (Ricks, 1978) and this would be compatible with the idea that a continual uptake of acetate irrespective of physiological and nutritional state would be required for the efficient metabolic functioning of these organs.

Additional evidence in support of the idea that large fluctuations in acetyl CoA synthetase activity of heart tissue do not occur is provided by the data of the calf experiment (Table 13). In contrast to the fatty acid activating ability of liver and kidney tissue substantial acetate activating ability (mμmoles/min/mg protein) is present in heart mitochondria both in the fetus (20±5) and at birth (18±4). Enzyme activity did decrease after birth (9±4 at 14 days of age) and then increased with age (33±8 at 120 days of age). However, neither a significant effect of diet on acetate activation at 60 or 120 days of age (Table 13) nor a significant correlation of blood acetate concentration to acetate activating ability was detected. Observations which suggest that acetyl CoA synthetase activity develops irrespective of whether the animal has a system of metabolism based on glucose (liquid diet fed group) or on glucose and fatty acids (normal fed group).

Although acetate concentration in peripheral blood is not correlated with heart mitochondrial acetyl CoA synthetase activity, it is possible that placental transfer of acetate from the maternal blood stream may influence enzyme activity. Fetal plasma acetate concentration (mM) (Table 12) is high (.627±.046) relative to the level at 14 days of age ($P < .05$) and although an endogenous production of acetate by fetal liver cannot be ruled out this seems unlikely on the basis that blood levels of acetate (mM) do decrease after birth to .2±.043 at 14 days of age (Table 12). Fetal acetyl CoA synthetase activity (μmoles/min/mg protein) is also high (Table 13) relative to the level at 14 days of age (20±5; 9±4 respectively). Fetal heart tissue, unlike many other fetal tissues, is functional early in gestation and may obtain its energy by the oxidation of acetate. It may be that acetate from the maternal blood stream induces enzyme activity *in utero*. If this is so, it may explain why the mitochondrial acetate activating ability of peripheral tissues in adult ruminants and non-ruminants is similar. A fact which has led Ballard (1972) to suggest that no specific adaptation to the increase in volatile fatty acids in ruminants has occurred at the level of acetyl CoA synthetase. It may be that in non-ruminants acetate from the intestines induces acetyl CoA synthetase activity. Levels of acetate in the blood of the ruminant fetus are similar (.6 mM) to the levels of acetate in the peripheral blood (.6 mM) of the fasted non-herbivore

(Ballard 1972).

Groot et al., (1976)have found that cytosolic forms of acetyl CoA synthetase do not occur in non-ruminant heart and kidney tissues to any significant extent. They have postulated that an active mitochondrial form of the enzyme only is required for the aerobic oxidation of acetate to provide ATP. In contrast, ruminants do contain a cytosolic form(s) of the enzyme (Table 14) and it is postulated that this may be adaptation which has occurred in response to the increased production of rumen volatile fatty acids and decreased absorption of glucose. The enzymes would allow the trapping of acetate in a given tissue against a concentration gradient. Evidence in support of this hypothesis has been obtained from the calf experiment. Cytosolic heart acetate activation increased with age (Table 14) and was significantly lower ($P < .05$) when animals were fed a liquid diet at 60 and 120 days of age than when animals were fed solid feed. In addition, a significant correlation ($r=.8438$, $P < .01$) was obtained for plasma acetate concentration with cytosolic acetate activation. These facts would indicate that cytosolic enzyme activity is influenced in some way by the increased rumen production of volatile fatty acids. It is concluded that cytosolic acetate activation may be a mechanism which the ruminant animal has evolved to trap increased amounts of acetate for subsequent use as an energy source, to compensate for the decreased amounts of energy that is can derive from glucose.

Volatile Fatty Acid Activating Enzymes of Liver Tissue

Purification of the fatty acid activating enzymes from liver mitochondrial tissue demonstrates for the first time that a distinct propionyl CoA synthetase with a high specificity for propionate but not the other short chain fatty acids (Figures 18 and 20, Table 8) is present in the bovine. Although a substantial purification of this enzyme was made (Table 7) the preparation was not homogeneous as evaluated by the technique of polyacrylamide gel electrophoresis (Figure 21). However, the partially purified enzyme was not contaminated by other short chain fatty acid activating enzymes since the Eadie-Scatchard plot of the kinetic data yielded straight line plots (Figures 26, 27 and 28). It is believed that a homogenous preparation could be attained by chromatography on DEAE-23 cellulose, calcium phosphate gel, phosphocellulose and 5'-AMP-Sepharose 4B (Figures 17, 18, 19 and 20).

On the basis of a number of observations it is believed that this propionate activating enzyme is similar to the propionyl CoA synthetase isolated from guinea pig liver mitochondria by Groot (1976) and from sheep liver by Latimer (1967). The substrate specificities are similar, with maximal activation on propionate followed by acrylate (Groot, 1976). Furthermore, the K_m's for propionate, ATP and coenzyme A are of similar orders of magnitude (Figures 26, 27 and 28) to those obtained by these

workers.

As a result of activation by propionyl CoA synthetase the propionate is trapped within the mitochondrion as the coenzyme A derivative. It seems reasonable to suppose that this enzyme, as the first committed step in the sequence of reactions leading to the synthesis of glucose, plays a major role in the control of the process. The K_m of the partially purified enzyme for propionate is 1.3×10^{-3}M (Figure 26). The concentration of propionate in portal blood can range from 0.3 mM to 2 mM depending on both the type of feed and time after feeding (Cook and Miller, 1965; Chase et al., 1977). Since propionate can diffuse readily through cellular membranes the propionate concentration in the cytoplasm would be within the same range as the concentration in portal blood. Thus, the concentration of propionate reaching the mitochondria may be within the range of the K_m of the enzyme i.e 0.3×10^{-3}M to 2×10^{-3}M. As a result the metabolic pathways of liver mitochondrial propionate metabolism must be regulated very simply by availability (concentration in portal blood) of substrate. A reduction in propionate concentration in portal blood will result in a decrease in the activity of the enzyme since it is not saturated with substrate. This will result in a decreased flux of propionate through the pathways of propionate metabolism such as gluconeogenesis. Small fluctuations in the concentration of propionate in portal blood will cause relatively large changes in the amount of glucose

synthesized from propionate. Feeding rations which yield higher levels of ruminal propionate or utilizing feed additives such as monensin which stimulate an active propionic acid fermentation should rapidly increase the conversion of propionate to glucose (within seconds). This would be equivalent to a fine control on the system since once the propionate has been absorbed into the portal blood rapid changes in the conversion of propionate to propionyl CoA and thus to glucose would occur.

The results of the calf experiment (Table 17) show that mitochondrial propionate activation is low in the fetus and newborn calf and increases with age. Enzyme activity at birth was significantly lower ($P < .05$) than enzyme activity at 60 and 120 days of age for animals fed solid feed. In general such increases in enzyme activity over a period of days are usually due to an increase in synthesis of new enzyme protein (Cook, 1978). Although the increase in propionate activation which occurs as the animal matures may not be solely due to an increase in propionyl CoA synthetase because the butyrate activating fraction also activates propionate (Table 9) it is likely that propionyl CoA synthetase does increase with age since the affinity of the butyrate activating fraction for propionate is low. It is, therefore tempting to speculate that the increase in propionyl CoA synthetase with age may be due to substrate induction since enzyme activity in animals maintained on a liquid diet was lower (Table 17)

than the activity in animals fed the solid feed and a positive correlation coefficient (Table 19) for blood acetate concentration with propionate activating ability within the mitochondria ($r=.7650$, $P < .02$) was obtained. It is assumed that there is a positive relationship between peripheral levels of plasma acetate and rumen production of volatile fatty acids and therefore a relationship between plasma acetate levels and portal propionate concentration. If this is true, the data would indicate that as portal propionate levels increase then so also does propionate activating ability within the mitochondria (Table 17 , Table 12). Lack of propionate activating ability at birth would be expected since very little propionate would be transferred from the maternal blood stream to the fetus. Only acetate is present in significant quantities in the peripheral blood of the dam.

Thus, feeding ingredients favorable to an active rumen propionic acid fermentation may not only control propionyl CoA synthetase activity *per se* but may also induce the synthesis of new enzyme protein in liver tissue and thus increase the potential for glucose synthesis from propionate. Preliminary observations from this laboratory reinforce this concept. Propionate activation of liver mitochondrial tissue appears to increase as lactation progresses and this may well be due to increased grain feeding practiced at this time which results in increased propionate levels in the rumen.

This has practical implications for the dairy industry. By suitable dietary manipulations propionyl CoA synthetase activity could be induced and many of the problems facing the high producing dairy cow alleviated. For example, it has been suggested (Young, 1977) that the milk yield of high producing cows is limited by glucose availability. Moreover, many such cows are susceptible to the metabolic disease ketosis during the first few weeks after parturition. The disease is thought to be associated with a lack of gluconeogenic precursors. Blood glucose levels fall and in compensation excessive mobilization of body fat occurs. The problem is compounded by the animal going off feed. If such cows could be identified the problem might be avoided by suitable dietary manipulation to increase rumen propionate levels prior to parturition such that propionyl CoA synthetase is induced and glucose synthesis can occur at high rates e.g. feed monensin 30 days prepartum. Increased grain feeding for long periods prior to parturition must be avoided however, because the excess energy is diverted into body fat; the animal becomes obese with subsequent calving problems and decreased milk production.

Maximizing propionate incorporation into glucose early in lactation is beneficial for other reasons than those outlined above. For the first few weeks after parturition the cow will be in a negative energy balance. To meet her glucose requirement substantial quantities of body protein will be mobilized. If propionate incorporation

into glucose could be increased then breakdown of body protein could potentially be decreased; an obvious advantage to an animal under conditions of metabolic stress such as lactation.

Cytosolic propionate activation in liver tissue increased with age (Table 18) but no effect of diet or correlation with blood acetate level was obtained. It is not clear therfore, what parameters control the propionate activation of the cytosol although the fact that this activity is absent in the rat (Scholte and Groot, 1975) might suggest that this may be an evolutionary adaptation which has occurred in the ruminant to allow these animals to trap propionate in the cytosol.

For a number of reasons the butyrate and valerate activation of liver mitochondrial suspensions cannot be accounted for by a single enzyme although it eluted as a single component on calcium phosphate gel (Figure 20). Firstly, the Eadie-Scatchard plots of kinetic data (Figures 29, 30, 31 and 32) gave qurvilinear plots indicating the presence of more than one molecular speicies with volatile fatty acid activating properties. Secondly sucrose density centrifugation (Figure 24) of the butyrate activating fraction indicated the presence of more than one molecular species with volatile fatty acid activating properties and lastly, Groot (1976) working with the fatty acid activating enzymes of guinea pig mitochondria could separate not only

a propionyl CoA synthetase as described above but also a medium chain fatty acid activating enzyme (classical butyrl CoA synthetase first described by Mahler et al. (1953)) with maximal activity on hexanoate followed by octanoate and butyrate and a salicyclate activating enzyme with maximal activity on benzoate and hexanoate with lower activity on octanoate and butyrate. The butyrate activating fraction isolated from bovine liver mitochondria activated both benzoate characteristic of the salicyclate enzyme and octanoate characteristic of the medium chain acyl CoA synthetase. It therefore probably is composed of two enzymes which can activate butyrate. It is believed that these two enzymes could be separated by phosphocellulose chromatography (Groot, 1976). On the basis of the sucrose density centrifugation experiment (Figure 24) it is likely that one enzyme migh activate primarily butyrate (butyrl CoA synthetase?) and the other primarily valerate (valeryl CoA synthetase?).

Groot (1976) has suggested that in the guinea pig these two enzymes may be involved in the initiation of medium chain fatty acid oxidation. They may also serve a role in the excretion of aromatic carboxylic acids by activation followed by conjugation with glycine (Killenberg et al. 1971; Forman et al., 1971). In the ruminant another role might be to remove all of the butyrate, which has escaped metabolism to β-hydroxybutrate by the rumen epithelium, from portal blood. Such a role would be compatible

with the observation that little butyrate appears in peripheral blood and must therefore be taken up by liver. Acetate is excluded as an energy furnishing substrate in ruminant liver. It is proposed that butyrate replaces this function of acetate in ruminant liver.

Since liver propionyl CoA synthetase has a low K_m (1.3×10^{-3}M) and high affinity for propionate (Figure 26) and the liver butyrate activating fraction has a low affinity for propionate (Figure 29) these enzymes may function in a manner analogous to glucokinase and hexokinase. At low or normal substrate concentrations propionyl CoA synthetase can activate all the propionate. However, when larger amounts of propionate are presented to the liver, the butyrate activating enzyme becomes important in propionate activation, to insure that all propionate in portal blood is taken up by the liver for glucose synthesis.

Data from the calf experiment shows (Table 17) that both butyrate and valerate mitochondrial activation are low at birth and increase with age ($P < .05$) as the animal becomes a ruminant. Although there was a significant correlation of blood acetate with both butyrate and valerate activation ($r=.8034$, $P < .01$; $r=.7177$, $P < .05$ respectively) no effect of diet could be detected. Therefore it is not clear whether these enzymes are influenced by the metaboic status of the animals. Certainly the low activity at birth would be compatible with a lack of placental transfer of butyrate or valerate. These acids are known to be low

in concentration in the peripheral blood of the dairy cow.

Cytosolic forms of the enzymes activating butyrate are absent in the rat (Scholte and Groot, 1975). Cytosolic forms activating butyrate are present in the bovine (Table 18). These forms are low at birth and increase with age. It is therefore, possible that this is an adaptation which has occurred in the ruminant to enable it to utilize more efficiently rumen derived volatile fatty acids as alternate substrates to glucose. However, no effect of diet or correlation with plasma acetate levels for either the butyrate or valerate activating ability in the cytosol could be detected.

The data explain the well known fact that ruminant liver does not oxidize significant quantities of acetate or use this as a substrate for lipogenesis. Mitochondrial and cytosolic acetate activating ability were low (Tables 17 and 18). In addition, acetyl CoA synthetase could not be isolated from liver mitochondrial extracts (Figure 17). Thus, ruminally derived acetate passes from the portal blood to the peripheral blood with little intermediary metabolism in liver. The physiological basis for this phenomenon is probably twofold. Firstly, it ensures that an energy furnishing substrate, acetate, is made available to peripheral tissues such as kidney and heart which may required it when alternative substrates such as glucose are in short supply. Secondly, it ensures that no synthesis of lipid can occur in the cytosolic fraction of ruminant liver via acetate activation to acetyl CoA and subsequent synthesis into long chain

fatty acids. Since glucose is also precluded as a carbon source for fatty acid synthesis in ruminant liver (Ingle et al., 1972b) this allows the primary role of ruminant liver to be synthesis of glucose and not fat i.e. gluconeogenesis can occur at all times. Gluconeogenesis and lipogenesis are processes which compete for ATP and carbon skeletons and thus cannot occur at maximal rates at the same time in the same organ (Tepperman and Tepperman, 1970). In the non-ruminant both cytosolic and mitochondrial acetyl CoA synthetases are found. In these animals it is not as important that acetate be excluded from mitochondrial metabolism in liver tissue since glucose is available as an energy furnishing substrate for peripheral tissues. Moreover, gluconeogenesis does not occur continuously. Fatty acid synthesis and gluconeogenesis can occur in liver because they are separated in time. This work shows that in ruminant, liver metabolism has become adapted to low blood glucose concentrations, not only by loss of the citrate cleavage enzyme (Hanson and Ballard, 1967, 1968) but also by loss of the mitochondrial and cytosolic forms of acetyl CoA synthetase. These modifications ensure that no metabolic process competes with gluconeogenesis in these animals.

Volatile Fatty Acid Activating Enzymes of Kidney Tissue

Kidney tissue is unique in that it contains an enzyme similar to the acetyl CoA synthetase found in heart and mammary tissue and enzymes similar to those characteristically found in liver mitochondrial tissue i.e. a propionyl CoA

synthetase and a butyrate activating fraction (Figures 33 and 35).

Since acetate is present in significant quantities in peripheral blood the presence of acetyl CoA synthetase appears reasonable. The enzyme enables kidney tissue to utilize acetate taken up from the blood, as a source of ATP necessary for maintaining its excretory function, and thus spares the action of glucose. Animals on a liquid diet (metabolism based on glucose) were deficient in acetyl CoA synthetase activity in the mitochondria (Table 15). Thus an active acetyl CoA synthetase may be an adaptation which has occurred in response to an increase in ruminally derived volatile fatty acids. However, in contrast to the situation found in heart tissue mitochondrial acetyl CoA synthetase activity was low at birth (Table 15) and since placental transfer of acetate probably does occur (Table 12) enzyme activity may not be controlled simply by substrate induction. These observations are probably related to the fact that fetal heart tissue is physiologically active early in gestation whereas the kidneys remain non-functional. The mother performs the necessary excretory functions required by the fetus by placental transfer of fetal wastes to the maternal blood stream.

The presence in kidney and liver of apparently similar enzymes activating propionate (Figure 35; Table 11) may be related to the gluconeogenic capacity of these organs. For example, the activity of these enzymes increases in liver

and kidney (Table 15 and 17) under conditions where glucose synthesis is required (animals on solid feed) and is lower when glucose is supplied from the diet as in the liquid fed group. However, the specific roles for these enzymes in the two organs cannot be identical. The reason for this is that propionate is only present in peripheral blood at very low concentrations (Cook and Miller, 1965).

The K_m of kidney propionyl CoA synthetase for propionate is of the same order of magnitude as that of the liver enzyme (2.5×10^{-3}M and 1.3×10^{-3}M respectively). At physiological concentrations of substrate, enzyme activity would be negligible. Therefore, a specific role comparable to that of liver for uptake of C_3 units from the blood for incorporation into glucose, is unlikely unless the apparent K_m is changed *in vivo* by the action of some activator yet to be identified. If this is the case, then this might be a mechanism which the ruminant animal has evolved to ensure that all C_3 units available can be trapped and converted into glucose. Another possibility is that propionyl CoA synthetase plays a role as yet undefined in amino acid catabolism. Certain amino acids are degraded via propionate. Although it is generally accepted that the intermediate involved is already in the coenzyme A form it is possible that propionyl CoA synthetase plays a role in the process. Lower enzyme activity in liquid diet fed animals (Table 15) than solid fed animals would be compatible with this theory because in the first group less glucogenic amino acids would

be catabolized for synthesis of their carbon skeletons into glucose, as glucose would be provided in the diet. Lastly, the physiological significance of a propionyl CoA synthetase in kidney mitochondria may be to activate substrates as yet unknown. Although no data is available on the rate of gluconeogenesis in ruminant kidney it is postulated that the kidneys play a major role in the synthesis of glucose comparable to that known to occur in the non-ruminant on prolonged starvation(Owen et al., 1969) and that propionyl CoA synthetase plays an important role, as yet undefined in this process.

Butyrate is not present in peripheral blood and therefore it is unlikely that the physiological significance of the mitochondrial butyrate activating fraction is in removal of C_3 and C_4 units from the blood unless unknown activators act in vivo to change the kinetic properties of the enzyme(s). A more likely role for this fraction would be in the β-oxidation of medium chain fatty acids. Kidney tissue is known to be metabolically very flexible; using glucose, fatty acids, amino acids and ketone bodies as energy furnishing substrates. The reason that liquid diet fed calves had lower enzyme activity (Table 15) ($P < .001$), than animals fed solid feed would be related to the increased use of fatty acids as energy furnishing substrate, relative to glucose in the latter case.

Volatile fatty acid activation of cytosolic fractions of kidney tissue was low at birth and increased with age

(Table 16). However, no effect of diet or correlation of blood acetate level with fatty acid activation was detected. It would seem that the increase in fatty acid activating bility which occurs after birth, occurs irrespective of the nutritional and metabolic status of the animal. Comparable fatty acid activation is absent in the cytosolic fractions of kidney tissue in the rat (Scholte and Groot, 1975) suggesting that the presence of such activity may be characteristic of ruminant animals.

CONCLUSIONS

Based on purification studies the reasons for the different volatile fatty acid activation patterns demonstrated by bovine heart (acetate, propionate), kidney (acetate, propionate, butyrate, valerate), and liver (propionate, butyrate, valerate) mitochondrial tissue have been determined. The different tissues contain different acyl CoA synthetases, enzymes responsible for trapping volatile fatty acids as the coenzyme A derivatives, with overlapping substrate specificities (Figure 39).

An enzyme (acetyl CoA synthetase), activating acetate and propionate, has been purified to homogeneity from heart tissue. The enzyme is a glycoprotein of apparent molecular weight 67,500 composed of a single polypeptide chain and is relatively stable compared to other acyl CoA synthetases. Part of the propionate and all of the acetate activation of kidney cortex tissue can also be accounted for by a similar enzyme. Qureshi (1971) and Stamoudis (1974) have demonstrated that acetyl CoA synthetase is present in lactating mammary mitochondrial tissue. The enzyme resembles that isolated from heart tissue in the present investigation. Many of the extra-hepatic tissues of the ruminant, therefore, contain an enzyme, acetyl CoA synthetase, which activates acetate and propionate, The presence of such an enzyme

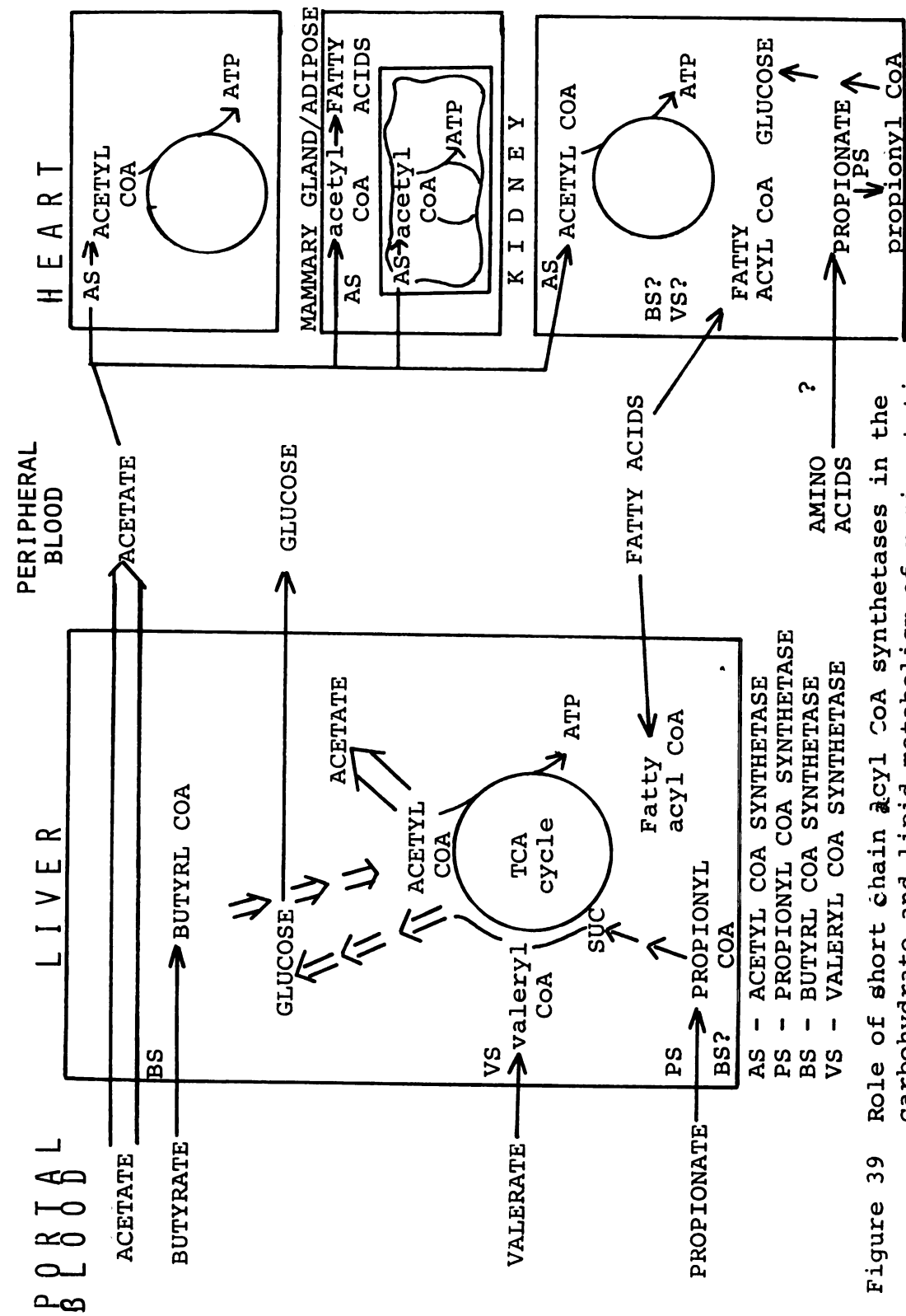


Figure 39 Role of short chain acyl CoA synthetases in the carbohydrate and lipid metabolism of ruminant tissues

within the mitochondrial matrix is assumed to be related to the ability of various tissues to use acetate as a major energy furnishing substrate for tissue metabolism (Figure 2). Only acetate and not propionate is present in any significant quantity in peripheral blood. Propionate activation by acetyl CoA synthetase may not therefore, be of any physiological significance in peripheral tissue.

Based on kinetic studies, it is concluded that acetate uptake by mitochondrial fractions of the peripheral tissues in the ruminant is probably not regulated by substrate availability. The K_m of heart acetyl CoA synthetase is 1.79×10^{-4}M and the K_m of the mammary gland enzyme is 6.1×10^{-4}M (Qureshi, 1971). The concentration of acetate in peripheral blood far exceeds these and so enzyme activity will be uneffected by any fluctuations in blood acetate that might be associated with changes in nutritional status.

Based on developmental studies, it is concluded that acetate uptake is controlled by the level of enzyme activity within a particular tissue. Acetate activation, and therefore acetyl CoA synthetase activity of kidney cortex mitochondrial tissue is negligible at birth but develops as the animal matures, providing the animal is fed solid feeds which can be fermented in the rumen to volatile fatty acids. In general, such increases over a sustained period of time, are due to an increase in synthesis of new enzyme protein. The development of acyl CoA synthetase activity is reduced when animals are fed an all milk diet which inhibits rumen

development. Thus, acetate will not be used as a major energy furnishing substrate in kidneys either in the newborn calf or in older animals in which rumen development has been blocked. Acetate utilization will become significantly more important as the animal shifts from a metabolism based on glucose to that of glucose and volatile fatty acids. This shift occurs as the rumen fermentation process becomes established. Although enzyme activity is associated with the presence of an active rumen fermentation it cannot be concluded that the enzyme is substrate inducible since enzyme activity is negligible in the fetus, even though significant quantities of acetate are present in fetal peripheral blood. The latter observation may be related to the fact that in the fetus the excretory function of the kidneys is provided by placental transfer of fetal excretory wastes to the maternal blood stream and thus the fetal kidneys are physiologically inactive until birth.

Developmental studies have demonstrated that acetyl CoA synthetase activity of heart tissue is substantial at birth and is subject to little variation as the animal matures and changes from the patterns of intermediary metabolism to the adult forms occur. It has been determined that acetate, unlike other volatile fatty acids is present in considerable amount in fetal blood. Since substantial acetate activation is present at birth, it may be that the enzyme is induced <u>in utero</u>. The ability to utilize acetate as a substrate for energy metabolism would be related to the functional activity of heart tissue early in

gestation.

This work demonstrates that the mitochondrial acetyl CoA synthetase characteristically found in many extra-hepatic ruminant tissues, is absent in liver mitochondrial tissue. It is postulated that this is an evolutionary adaptation which has occurred in ruminants (non-ruminants possess a mitochondrial form of acetyl CoA synthetase in the liver) to ensure that under conditions of low glucose availability an alternative energy furnishing substrate such as acetate is made available to those peripheral tissues which require them. It has been shown that acetyl CoA synthetase activity, rather than being present at birth and subsequently being lost as the shifts in energy metabolism occur, is absent at birth in the ruminant liver and never develops.

An enzyme, propionyl CoA synthetase, with a high specificity for propionate and acrylate, has been isolated and partially purified for the first time from bovine liver mitochondrial tissue. The enzyme has an apparent molecular weight of 73,400 and is relatively unstable compared to acetyl CoA synthetase. An enzyme with similar substrate specificity is present in kidney mitochondrial tissue. Heart tissue does not contain this enzyme. Thus, the enzyme appears to be a characteristic feature of only those tissues which are potentially gluconeogenic.

The ability of liver tissue to activate propionate is related to its capacity to synthesize glucose from ruminally derived propionate absorbed from portal blood.

Kinetic studies have elucidated the role of substrate availability in determining propionyl CoA synthetase activity of liver tissue. The K_m for propionate is $1.3x10^{-4}M$. This is withing the range of propionate concentration found in portal blood. It is concluded that as propionate levels in portal blood change in response to feeding, so enzyme activity will increase proportionally and more propionate will be taken up and incorporated into glucose.

The developmental studies have shown that another mechanism for potentiating propionate conversion to glucose may be to increase the level of enzyme activity. This is most likely the result of an increase in enzyme synthesis. Propionate activation in liver at birth is low and increases with age providing rumen fermentation is established. Moreover, a positive correlation of acetate concentration in peripheral blood and therefore propionate concentration in portal blood to propionate activation exists. It is therefore likely that a continuing increase in rumen propioante levels over sustained periods of time results, either by substrate induction, or by some mechnaism undefined, in increased levels of propionyl CoA synthetase activity. This observation is of economic importance to the dairy industry. It is concluded that by suitable dietary manipulations rumen propionate levels can be increased with the result that both the enzyme activity already present will be stimulated, and over longer periods an increase in total enzyme activity probably will occur as a result of enzyme synthesis. By

stimulating propionate conversion to glucose the milk production of high producing cows may be increased and the incidence of metabolic diseases such as ketosis may be decreased. Monensin, an activator of ruminal propionate fermentors, may provide beneficial effects in this way.

The data demonstrate that the activity of kidney propionyl CoA synthetase is strongly related to the presence of an active rumen fermentation. However, unless activators of the kidney enzyme act *in vivo* to change the apparent K_m of the enzyme for propionate, it is unlikely that the physiological function of this enzyme is to remove propionate from peripheral blood for incorporation into glucose since this acid is present in only marginal amounts in peripheral blood. A role in the amino acid catabolism of glucogenic amino acids is implicated.

The butyrate and valerate activating ability characteristic of both kidney and liver mitochondrial fractions has been accounted for, using purification techniques, by a butyrate activating fraction which although it exhibits some activity with propionate as a substrate is distinct from propionyl CoA synthetase. However, based on both kinetic studies and sucrose density centrifugation estimates of molecular weights, for the liver fraction, it cannot be considered one acyl CoA synthetase of broad substrate specificity. The fraction is composed of two acyl CoA synthetases of molecular weights 67,000 and 65,000. The former activating butyrate and the latter activating valerate.

This constitutes evidence for a separate butyrl and valeryl CoA synthetase in liver and probably kidney also.

The physiological function of the butyrate activating fraction in liver would be: (1) to remove any butyrate and valerate from portal blood that excapes metabolism by the rumen wall; butyrate could then be used as an energy source for liver function and valerate possibly as a glucose precursor, (2) to activate medium chain fatty acids for β-oxidation within liver tissue. Only the second function is applicable to kidney tissue since these substrates are present in only marginal amounts in peripheral blood.

The data would suggest that the ability to activate butyrate and valerate, like that for propionate, is associated with shifts in the patterns of inetermediary metabolism which occur when the young ruminant is fed diets that stimulate rumen development and fermentation. Developmental studies have demonstrated that the patterns of volatile fatty acid activation characteristically associated with the mitochondrial fractions of heart, kidney and liver are duplicated in the cytosolic fractions of these tissues. It may therefore be concluded that acyl CoA synthetases similar to those found in the mitochondrial fraction occur in the cytosol, i.e. an acetyl CoA synthetase in heart cytosolic fractions, a propionyl CoA synthetase and a butyrate activating fraction in liver cytosolic fractions. In addition, the presence or absence of cytosolic volatile fatty acid activating ability in these tissues at birth

corresponds to the pattern obtained for the mitochondrial fractions at birth. It is absent in liver and kidney but present in heart. Volatile fatty acid activating ability developed in liver and kidney tissue as the animal matured irrespective of nutritional and metabolic status.

The specific roles of the cytosolic acyl CoA synthetases remain to be established. It is postulated that these forms ensure that whatever volatile fatty acids are required by the mitochondria are trapped within the cytosol and subsequently transferred via the carnitine transport system to this organelle. A major role of these enzymes in fatty acid synthesis in liver tissue is excluded since lipid synthesis occurs primarily in adipose depots in ruminants.

In conclusion, the presence of particular acyl CoA synthetases within the mitochondrial and cytosolic fractions of heart, kidney, and liver tissue ensures that acetate is used primarily be extra-hepatic tissue as an energy source, and propionate and butyrate are removed by the liver the former for use as a substrate for gluconeogenesis, and the latter as an energy furnishing substrate or a carbon source for ketone body formation. The roles of these enzymes in ruminant metabolism are shown diagrammatically in Figure 39.

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