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FATTY ACID SYNTHESIS IN THE AMERICAN EEL, *ANGUILLA ROSTRATA* (LeSUEUR): TISSUE SITES, REDUCING EQUIVALENTS AND CARBON SOURCE(S)

by

Peter L. Aster

A Thesis

presented to the School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in the

Department of Biology

Ottawa, Ontario, 1981

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ABSTRACT

The tissue sites, reducing equivalents, and sources of carbon for de novo fatty acid synthesis were investigated in the immature American eel, *Anguilla rostrata* (LeSueur).

The rates of in vivo and in vitro incorporation of ^{14}C-acetate into lipids indicated that the liver and intestine are the tissues most actively involved in fatty acid synthesis. Neither red nor white muscle incorporated significant label into lipids.

The comparative ability of selected eel tissues to produce fatty acids was further evaluated by measuring the activity of cytoplasmic NADPH producing enzymes: isocitrate dehydrogenase (IDH), malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH), and 6-phosphogluconate dehydrogenase (6PGDH). The liver had the greatest capacity to produce reducing equivalents (NADPH), although significant activities were also noted in the intestine.

The influence of diet and food-deprivation on liver enzymes was examined. There were no differences in enzyme activities between eels fed beef-liver or fasted for 2 months. Eels fed worms had significantly greater G6PDH activity than those fasted for 4-6 months. Liver weight and hepato-somatic index decreased in fasted eels, but lipid content per gram of liver or muscle increased.

In the liver, NADP-IDH had the lowest Km(NADP) and the highest activity of the four cytoplasmic NADP-dehydrogenases. Only low activities of ATP-citrate lyase (CCE) and ME were found. Aconitase was present only in the cytoplasm and it had a Km(citrate) 20-times lower and an activity at least 7-times higher than CCE. These results indicate that carbon flow from extramitochondrial citrate is towards isocitrate, and that IDH

is a major source of reducing equivalents.

As a consequence of the enzyme studies, particularly at the citrate branchpoint, the hypothesis that synthesis of fatty acid carbon in eel liver utilizes ketogenic precursors rather than glucogenic precursors was tested with in vitro label experiments.

The rates of incorporation of the ketogenic precursors, ^{14}C-acetate and ^{14}C-acetoacetate, into fatty acids in eel liver slices were at least 4-times higher than rates of glucogenic precursor (^{14}C-lactate, citrate, aspartate, glucose) incorporation.

Tritiated water was used to measure rates of lipogenesis independent of carbon source. The mean rate of de novo fatty acid synthesis was 1.53 μmoles of $^{3}H_2O$ incorporated/g wet weight of liver/2 hr at 15°C. When ^{14}C-precursor and $^{3}H_2O$ (^{3}HOH) incorporation were compared in paired experiments, acetate and acetoacetate were incorporated into fatty acids at 70% of the rate of $^{3}H_2O$, while lactate and other glucogenic precursors did not exceed 10% of the $^{3}H_2O$ rate. Although lactate did not contribute a significant amount of fatty acid carbon, it was, nonetheless, required to obtain maximal rates of de novo lipogenesis.

The overall pattern of de novo fatty acid synthesis in eel liver is similar to that of ruminant adipose tissue and may be an adaptation to spare glucose and glucogenic precursors.

Food-deprivation for 4 months caused a 5-fold reduction in the in vitro rate of fatty acid synthesis. Additional periods of starvation reduced the rate still further, and at 9 months a 30-fold decrease in vivo was observed.

The oxidation of ^{14}C-oleate was measured to determine whether the increased lipid content of the liver in starved animals was due to

impaired lipid catabolism. Rates of oleate oxidation were at least 2-fold higher in starved compared to fed eels, and thus decreased fatty acid utilization was not the explanation for the higher lipid content.

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ABBREVIATIONS

G6PDH - glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

6PGDH - 6-phosphogluconate dehydrogenase (EC 1.1.1.44)

ME - malic enzyme (EC 1.1.1.40)

IDH - isocitrate dehydrogenase (EC 1.1.1.42)

CCE -(citrate cleavage enzyme) ATP-citrate lyase (EC 4.1.3.8)

MDH - NAD-malate dehydrogenase (EC 1.1.1.37)

Other abbreviations and/or EC numbers are presented in the text.

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INTRODUCTION

Fish lipids have been of considerable scientific interest for well over a half century, and much of this early work has been reviewed by Love (1970) and Shul'man (1974). Recent studies have examined the role of lipids in temperature acclimation (Hazel and Sellner, 1979), the effect of diets on lipid composition (Lin et al., 1977a,b), and the utilization of lipids during periods of energy depletion (Bilinski, 1974). Despite this continued interest, some of the most fundamental aspects of the uptake, synthesis, and utilization of lipids in fish tissues remain unclear (Patton et al., 1978).

I. LIPID METABOLISM IN ANGUILLIFORM EELS

Some of the most intensively studied fishes in terms of lipid composition and utilization of energy stores during starvation are the eels (*Anguilla* sp.), including the American, European, and Japanese species. This group of catadromous fishes has provided a fertile ground for physiological and biochemical studies. The immature, freshwater yellow American eel (*A. rostrata*) is a relatively lean fish, while the mature bronze of silver eel migrates seaward to spawn as a fatty fish. In all likelihood, it is upon these fat stores that this mature, non-feeding animal depends for energy to reach its spawning grounds in the southwest Atlantic. The American eel slowly increases its lipid content with maturation (personal observation), as do many other fishes (Love, 1970). It does not demonstrate seasonal fluctuations related to development or reproductive cycles (Shul'man, 1974), except during the yellow to silver transition just prior to its seaward

migration (Lewander et al., 1974). In addition, eels undergo a natural low temperature winter fast (Nyman, 1972), so an experimental fast, which is often used in studies of lipid synthesis, is not as abnormal a perturbation as in some other animals studied in this regard. Therefore, the eel represents a natural model for the study of lipid synthesis.

Changes in body composition including lipids due to the yellow-silver transformation and to starvation have been investigated primarily in the European eel, *A. anguilla*. Lewander et al. (1974) and Dave et al. (1974) found surprisingly few differences between older yellow eels (340 g) and newly transformed silver animals (420 g). The only changes found by Wills and Hopkirk (1976) in the yellow-silver transformation of *A. australis* were also quantitative rather than qualitative.

More informative, though, on occasion, confusing, are the studies of overwintering starvation in smaller (106-133 g) yellow eels by the Swedish group (Larsson and Lewander, 1973; Dave et al., 1975). Eels studied in the 1973 paper utilized liver triglycerides rather quickly, while muscle triglycerides were only utilized after prolonged starvation, concurrent with an increase in plasma free fatty acids (FFA). In the 1975 study both liver and muscle triglycerides decreased from 47 to 96 days of starvation, but plasma FFA, though variable, did not increase even after 164 days starvation. The utilization of triglycerides, rather than protein, for energy after moderate starvation had previously been shown in the work of Inui and Oshima (1966) on *A. japonica*. However, Lovern (1939) found that proteins were utilized during starvation in yellow *A. anguilla*, resulting in a relative (possibly absolute) increase in lipid content. Supporting this latter work is the finding of Renaud and Moon (1980b) that American eels increase

their utilization of protein for gluconeogenesis during starvation. The results of Lovern (1939) and Renaud and Moon (1980b) are consistent with those of Creach and Murat (1974) for carp (*Cyprinus carpio*), Kluytmans and Zandee (1974) for northern pike (*Esox lucius*), and Narayansingh and Eales (1975) for brook trout (*Salvelinus fontinalis*). These studies demonstrated no decrease in either the quantity of lipid reserves or the extent of label incorporation into lipids during fasting.

Despite the inherent interest in winter starvation and developmental phenomena, it is apparent that eels and other fish spend much of their lives neither starving nor transforming. It may, therefore, be of considerable value to define the general features of their lipid metabolism, particularly lipid synthesis, during the rest of their life cycle including periods of normal growth and feeding. This is of great importance, since the success of each animal in overwintering, migration, and reproduction is likely to be critically linked to its mechanism(s) of energy accumulation and storage.

II. LIPID SYNTHESIS

The de novo synthesis of lipids in the cytoplasm of vertebrate tissues requires (1) a carbon source for acetyl CoA, (2) reducing equivalents (NADPH) produced by one or more of four cytoplasmic dehydrogenases, glucose-6-phosphate dehydrogenase, G6PDH; 6-phosphogluconate dehydrogenase, 6PGDH; isocitrate dehydrogenase, IDH; and malic enzyme, ME; and (3) glycerol-1-phosphate for esterification.

The importance of different tissues to whole animal lipogenesis, and the pathways utilized to provide acetyl CoA and NADPH for the process varies in different organisms. For example, adipose tissue is the

major site of lipogenesis in ruminants (Bauman et al., 1976). Mice and rats appear to utilize both liver and adipose tissue for lipogenesis (Clark et al., 1977; Baker et al., 1978), while birds (Pearce, 1980) and man (Shrago et al., 1971) primarily use the liver.

In fish, where the fat storage areas may be diffuse (Tashima and Cahill, 1965; Vague and Fenasse, 1965), the liver actively produces lipids in several species (northern pike, Kluytmans and Zandee, 1974; Amazon catfish, Patton et al., 1978), and is probably the primary lipogenic organ (Lin et al., 1977a). However, other fish tissues do have the ability to incorporate labeled precursors (usually acetate) into lipids as well (goldfish muscle, Knipprath and Mead, 1968; rudd skin, Saxena and Zandee, 1971; goldfish brain, Leslie and Buckley, 1976).

The cytoplasmic location of the enzymes involved in fatty acid synthesis (Romsos and Leveille, 1974; Figs. 1A,B) requires that acetyl CoA, formed primarily in the mitochondria, be transported to the cytoplasmic compartment. In some lipogenic tissues (Fig. 1B) a carbon source such as acetate may enter the cell and be utilized directly for lipogenesis without first entering the mitochondria. Activated (CoA) substrates can not cross the mitochondrial membrane (Haddock et al., 1970), and a number of mechanisms for the transport of acetyl units from the mitochondria to the cytoplasm for fatty acid and sterol synthesis have been identified (Fig. 2; Pathways numbered in text below correspond to those numbers in Fig. 2). Acetyl carbon may be transported (1) by the tricarboxylate transporter linked to ATP-dependent citrate lyase step in the citrate cleavage pathway, (2) as acetate (Spencer and Lowenstein, 1962), (3) as N-acetylaspartate (D'Adamo et al., 1968; Patel and Clark, 1980), (4) as acetylcarnitine (Fritz and Yue, 1964), or (5) as

Figure 1A (*Upper*). Pathways of fatty acid synthesis in rat adipose and liver tissue (from Saggerson, 1980). Reducing equivalents (NADPH) are produced by the pentose phosphate pathway (1) and by malic enzyme (4). Fatty acid carbon is supplied by citrate cleavage enzyme (ATP-citrate lyase)(2). NAD-malate dehydrogenase (3) connects citrate cleavage with reducing equivalent production and is the first step in a transhydrogenation (NADH ⟶ NADPH) from oxaloacetate to pyruvate. The citrate cleavage pathway consists of steps (2), (3), (4).

Figure 1B (*Lower*). Pathways of fatty acid synthesis in ruminant adipose tissue (from Saggerson, 1980, based on Bauman, 1976). Fatty acid carbon is supplied by acetate which is activated in the cytosol by acetyl CoA synthetase (1). The citrate cleavage pathway does not operate due to negligible activities of citrate cleavage enzyme and malic enzyme (Fig. 1A, (2), (4)), and citrate formed in the cytoplasm is converted to isocitrate by aconitase (2). Reducing equivalents are provided by the pentose phosphate pathway (not shown) and by NADP-isocitrate dehydrogenase (3).

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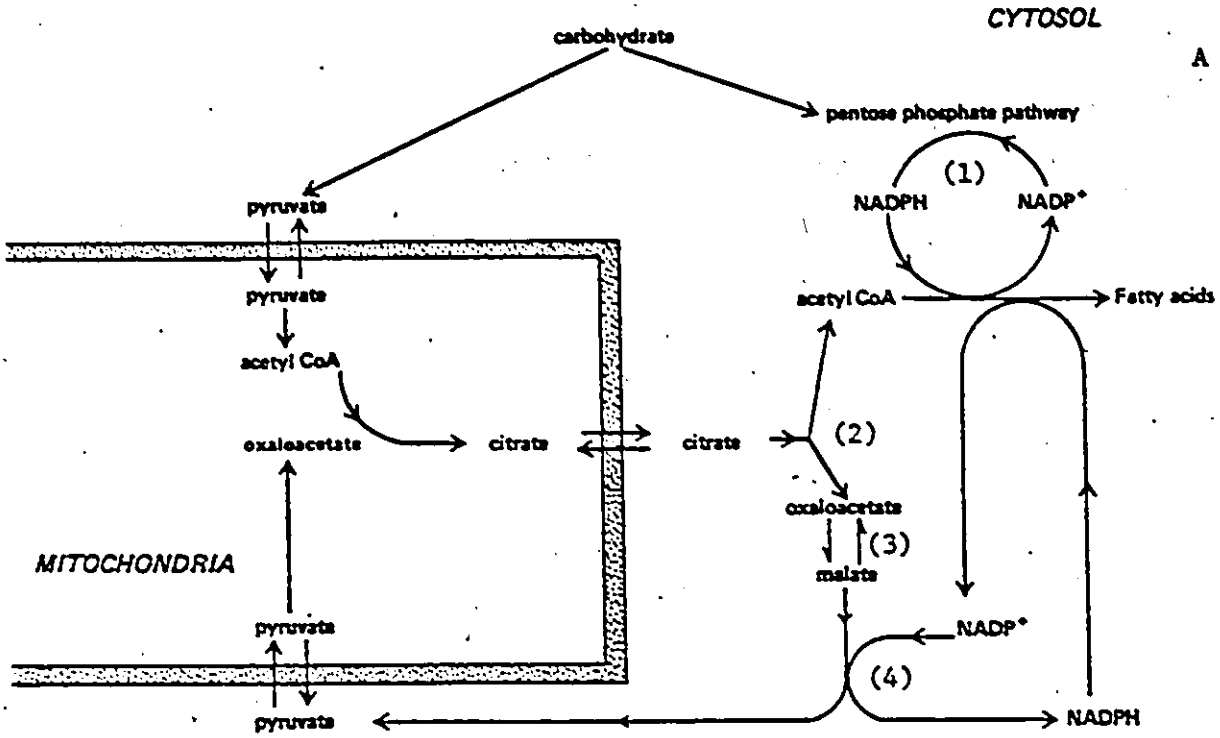
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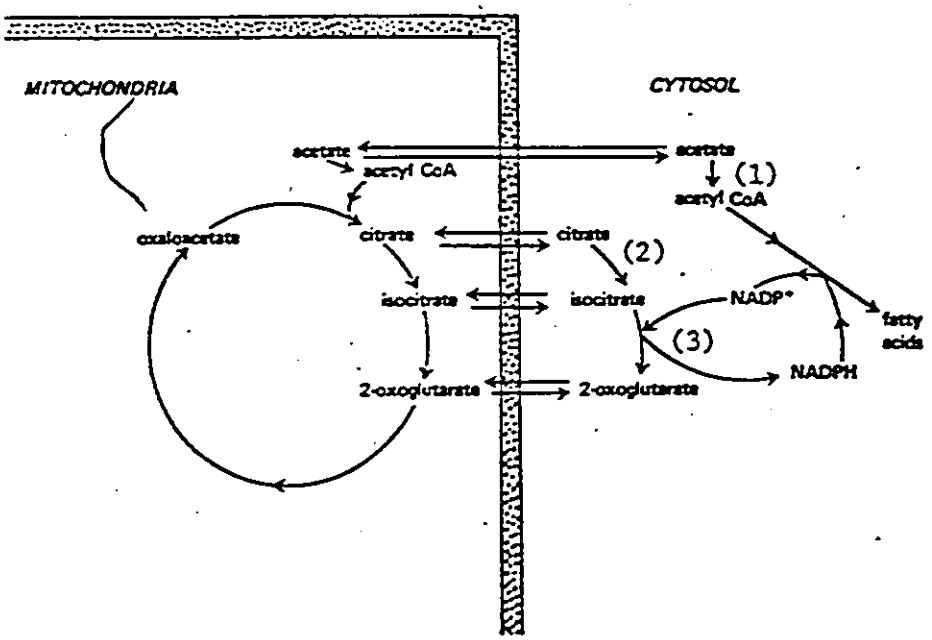
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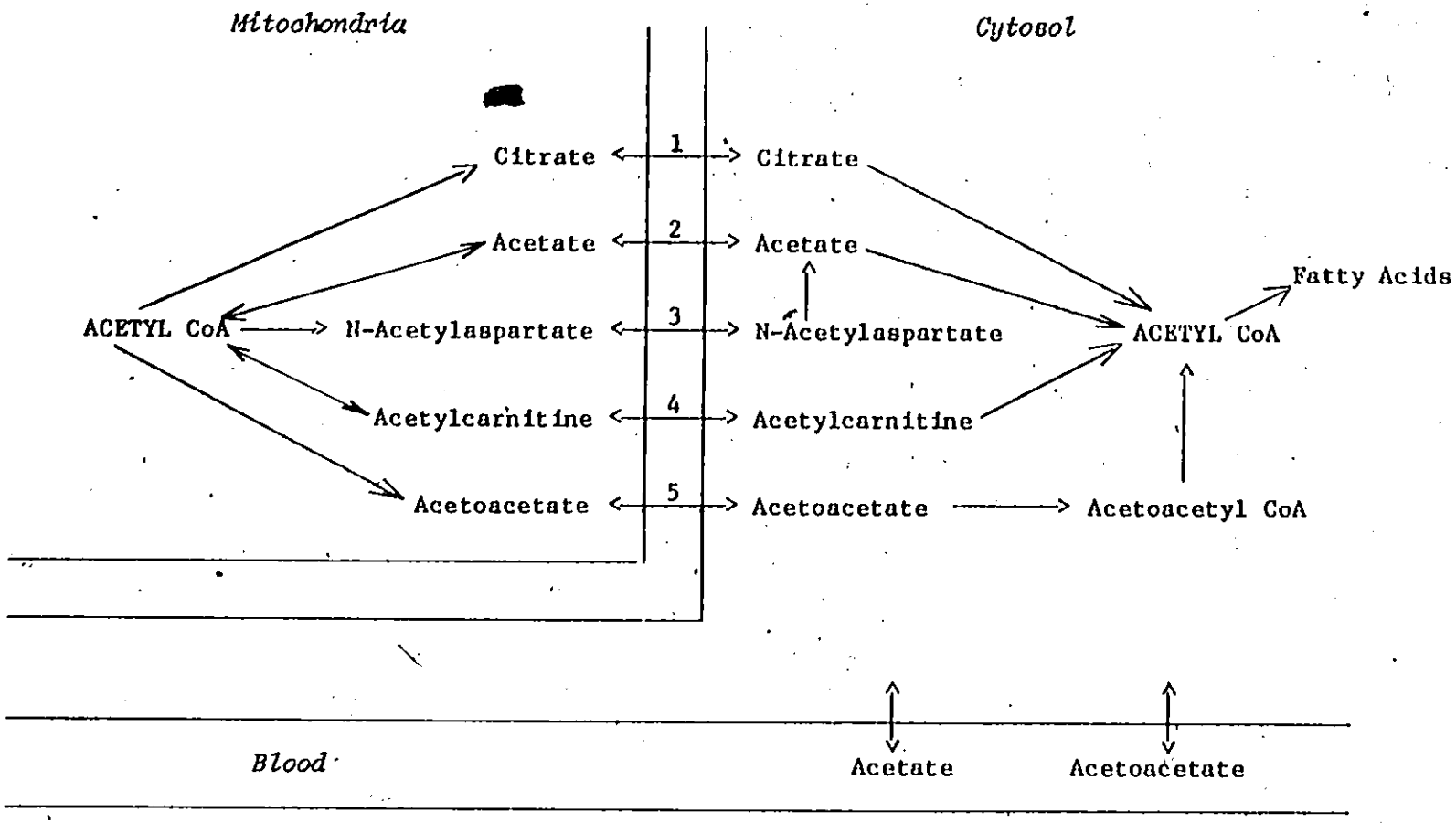


Figure 2. Pathways (simplified) for the transport of acetyl carbon from the mitochondria to the cytosol. See text for further details of numbered pathways.

acetoacetate (Rous and Favarger, 1973; Patel and Clark, 1980). The pathway utilizing N-acetylaspartate to transfer acetyl units appears to be restricted to brain tissue, where in vitro it does not account for more than about 20% of the acetyl units used for lipid synthesis (Patel and Clark, 1980). Similarily, acetylcarnitine, though formed in many tissues by carnitine acetyltransferase, has never been shown to account for more than a minor portion of the mitochondrial-cytoplasmic transfer of acetyl units for lipogenesis (Srere, 1965).

Acetoacetate is an important carbon precursor for lipid synthesis in the developing rat brain (Webber and Edmond, 1979) and may play a significant role in acetyl group transfer (Patel and Clark, 1980), although much of the acetoacetate used for lipogenesis is probably activated directly in the cytoplasm by acetoacetyl CoA synthetase after entering from the blood stream (Buckley and Williamson, 1973). Rat adipose tissue and mammary gland can also use acetoacetate as a minor source of lipid carbon, presumably through this cytoplasmic pathway (Robinson and Williamson, 1980).

The two primary sources of carbon for lipogenesis in mammalian tissues are citrate and acetate (Figs. 1A,B). Acetate is usually considered to be activated directly in the cytoplasm, based on the location of acetyl CoA synthetase primarily in that compartment in lipogenic tissues (Knowles et al., 1974). The citrate cleavage pathway is the major route for acetyl unit translocation when the carbon sources for lipogenesis are glucose, lactate, or glucogenic amino acids, as is the case in most rat and fetal ruminant tissues (Fig. 1A). In lipogenic tissues this pathway has the added advantage of producing reducing equivalents by a two-step transhydrogenation, from oxaloacetate to

malate to pyruvate where the hydrogen from NADH is transferred to NADPH (Fig. 1A). In monogastric mammals other than rats, the citrate cleavage pathway appears less important as a means of generating extramitochondrial acetyl CoA and this is correlated with an increase in the utilization of extramitochondrial acetate as a carbon source (Saggerson, 1974). The extreme example of an acetate rather than glucose or lactate based fatty acid synthesis is ruminant adipose tissue (Bauman, 1976; Fig. 1B). Unfortunately, there have been no studies of the source(s) of carbon for lipogenesis in fish tissues, and it is not known which, if any, of the mammalian models fish tissues resemble.

The pathways utilized to produce NADPH for fatty acid synthesis reflect the carbon flow, in part, and thus may be expected to differ in the two extreme mammalian lipogenic models; i.e., rat and ruminant (Figs. 1A,B). In rat liver and adipose tissue, the pentose phosphate pathway (G6PDH, 6PGDH; (1) in Fig. 1A) provides 50-80% of the reducing equivalents (Kather and Brand, 1975) and malic enzyme ((4) in Fig. 1A) provides the remainder. However, the virtual absence of malic enzyme and ATP-citrate lyase ((2) in Fig. 1A) in ruminants eliminates this pathway of reducing equivalent production (Fig. 1B). Furthermore, the absence of ATP-citrate lyase prevents the utilization of glucogenic substrates for the production of fatty acid carbon (Figs. 1A,B). In ruminants, both the pentose phosphate pathway and isocitrate dehydrogenase ((3) in Fig. 1B) are important in the provision of NADPH (Bauman, 1976).

Only two species of fish, both salmonids, have been investigated in regard to the supply of reducing equivalents, although numerous reports of individual dehydrogenases have been published (see Hazel and

Prosser, 1974). Baldwin and Reed (1976) have shown that all four cytoplasmic NADPH producing enzymes had similar activities in rainbow trout liver while no ATP-citrate lyase was detected. This latter, unusual finding, indicates that despite an active malic enzyme, the citrate cleavage pathway is inactive in trout liver. Lin et al. (1977a,b) have found all four NADPH producing enzymes plus ATP-citrate lyase to be active in coho salmon liver but much less active in mesenteric fat. Furthermore, high fat diets or fasting reduced the activity of the liver enzymes. They concluded that for coho salmon, as in rats, the pentose phosphate pathway and malic enzyme provide the reducing equivalents for lipogenesis.

The differences in tissue sites of lipogenesis and pathways utilized for NADPH production in closely related animals (Shrago et al., 1971) or in fish sharing the same habitat (Aster, 1976) suggests that the studies of Baldwin and Reed (1976) and Lin et al. (1977a,b) may not be generally applicable, and that more detailed studies in this regard are required.

The object of this study, therefore, is to provide data which could form the basis of a model of de novo fatty acid synthesis in a non-fatty fish, the yellow or immature American eel. The study is divided into essentially three parts.

1. It is initially necessary to determine which tissues are most actively involved in fatty acid synthesis. More specifically, the hypothesis will be tested that the liver is the most important tissue in this regard, as has been previously suggested for fish by Vague and Fenasse (1965), Tashima and Cahill (1965), Lin et al. (1977a,b), and others.

2. Based on the assumption that the carnivorous-omnivorous diet of eels (Sinha and Jones, 1975) and most fish is low in carbohydrates (Love, 1970), it would be predicted that there would be a "need" to spare glucose for energy utilization in those tissues that require it (white muscle, brain). If so, fatty acid synthesis would utilize carbon sources other than those which are directly glucogenic (e.g. lactate and alanine); for example, the ketogenic substrate acetate. Thus, of the two extreme mammalian models of de novo fatty acid synthesis (rat and ruminant adipose), it is predicted that this process in eels would more closely resemble the ruminant model.

This prediction will be tested in two stages. First, the potential of the different pathways of cytoplasmic NADPH production to supply reducing equivalents for fatty acid synthesis is evaluated by considering total enzyme activities, Michaelis constants, and responses to different diets and starvation. Second, the importance of glucogenic vs. ketogenic carbon sources will be assessed by looking at the enzymes of the extra-mitochondrial citrate branchpoint (ATP-citrate lyase and aconitase), and the results of these enzyme measurements confirmed by directly testing different precursors for lipogenesis using labeled substrates and tritiated water under controlled in vitro conditions.

After determining the sources of reducing equivalents and lipid carbon, an attempt will be made to speculate on the manner in which this "model" can be integrated with what is known about hepatic gluconeogenesis and the tricarboxylic acid cycle in eels (Hayashi and Ooshiro, 1979; Moon and Ouellet, 1979; Renaud and Moon, 1980a,b) and on its applicability to other fish and lower vertebrates.

3. Finally, since the yellow eel undergoes a low temperature

winter fast, the effects of food deprivation on the synthesis and utilization of lipids will be considered. In addition, the energy reserves of these animals will be evaluated and some speculations made on the role of lipid metabolism.

MATERIALS AND METHODS

I. ANIMALS AND DIETS

Immature yellow eels, *Anguilla rostrata* (LeSueur), between 50 and 180 g in weight, were captured by dip-net during their upstream migration in the St. Lawrence River at the W.B. Saunders Hydroelectric Dam, Cornwall, Ontario during August and September of 1976-1979. Water temperature ranged from 16-22°C. The eels were transported to the laboratory and were kept in 150 liter tanks of 14-16°C dechlorinated running water.

The fish were either killed within one week of capture (designated "freshly caught"), or maintained in the laboratory for up to two years. Those eels held in the lab were either deprived of food or fed beef liver or worms 4 to 5 times per week. The composition of the beef liver and worm diets is shown in Table 1. The lower fat and lower energy contents per gram of the earthworms were major differences between the two diets. However, the eels fed on the live prey more readily than on small pieces of liver, which probably compensated for these differences. All experiments performed in the fall of 1978 or later used eels fed worms. These eels would not eat pelleted fish rations. Somewhat arbitrarily, those eels deprived of food for less than 6 months are termed "fasted" eels, since this time period of food-deprivation may be encountered during a natural, winter fast, albeit at lower temperatures. Those eels deprived of food for more than 6 months are termed "starved". In general, the latter term is more inclusive and is used when food deprivation is discussed. For most experiments, eels averaging just over 100 g in weight were used.

TABLE 1.

Composition of eel diets

	Beef liver[1]		Earthworms[2]	
	% Weight	% Energy	% Weight	% Energy
Glycogen[3]	0.6	1.9	0.6	3.5
Protein[4]	18.5	61.5	10.4	65.4
Lipid[5]	4.9	36.6	2.2	31.1
Other (Ash, NPN)	8.1	-	11.6	-
Water	67.9	-	75.2	-

[1]Ottawa Beef Co. Ltd., Ottawa, Ont. [2]*Lumbricus* sp., Eileen Cordes, London, Ont. [3]Determined by the method of Renaud and Moon (1980a). [4]Determined by the method of Maddy and Spooner (1970). [5]Determined by the method of Folch et al. (1957).

II. ENZYME STUDIES

A. Preparation of tissue homogenates.

Eels were decapitated and the tissues were immediately removed and weighed. Red muscle and white epaxial muscle were excised from an area 2 to 10 cm posterior to the anus.

Tissues in 5 to 10 volumes of ice cold buffer (50 mM Tris-HCl, pH 7.5 at 20°C, 225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA) were homogenized with several short low speed bursts of a Polytron PCU-2 tissue homogenizer (Brinkmann), and centrifuged at 700 x g for 10 minutes in a Sorvall RC-2B refrigerated centrifuge at 0 - 4°C. The supernatant fraction was filtered and recentrifuged at 8000 x g for 10 minutes. The pellet from this operation has been shown to contain intact, coupled mitochondria (see Moon and Ouellet, 1979). This pellet was washed twice, frozen and thawed, then homogenized to release mitochondrial enzymes. The supernatant from the 8000 x g spin was recentrifuged at 39000 x g for a minimum of 30 minutes, and this supernatant was used directly for assays of the cytoplasmic enzymes. Homogenates were usually frozen (-20 to -30°C) one to five days before analysis. Preliminary tests established that activity losses were negligible with this procedure.

B. Enzyme assays.

Enzyme activity in the homogenate fractions was assayed by recording the reduction of NADP or oxidation of NADH at 340 nm with a Unicam SP1800 recording spectrophotometer. A Haake model FK temperature controller was coupled to the cuvette holder to maintain constant temperature. All assays were performed at 20°C in a final concentration

of 50 mM Tris-HCl buffer, pH 8.0, and a total volume of 3.0 ml. The reaction mixtures were selected to give optimal activities with liver homogenates, and were as follows (final cuvette concentrations):

Isocitrate dehydrogenase. 5 mM $MgCl_2$, 0.3 mM NADP, and 0.5 mM threo D_s isocitrate.

Malic enzyme. 1 mM $MnCl_2$, 0.3 mM NADP, and 5 mM malate.

Glucose-6-phosphate dehydrogenase. 5mM $MgCl_2$, 0.3 mM NADP, and 1 mM glucose-6-phosphate.

6-Phosphogluconate dehydrogenase. 5 mM $MgCl_2$, 0.3 mM NADP, and 0.5 mM 6-phosphogluconate.

NAD-malate dehydrogenase. 0.15 mM NADH and 0.5 mM oxaloacetate.

Aconitase. 10 mM $MgCl_2$, 0.5 mM NADP, 5 mM citrate, and excess isocitrate dehydrogenase (pig heart). Addition of ferrous ammonium sulfate did not enhance the activity.

ATP-citrate lyase. 20 mM $MgCl_2$, 0.1 mM NADH, 1 mM dithiothreitol, 0.4 mM CoA, 10 mM ATP, 20 mM citrate, and excess malate dehydrogenase (pig heart).

Endogenous NADP reduction or NADH oxidation, when significant, was allowed to proceed until a constant, negligible rate was recorded, usually within 3 to 5 minutes. Enzyme specific reactions were initiated by the addition of substrate. Appropriate blank cuvettes were used when necessary.

Kinetic parameters were determined from double-reciprocal plots or plots of 1/v vs. inhibitor concentration (Dixon plots, Dixon and Webb, 1964, p 357) by least squares linear regression. Michaelis constants, or Km values, are the substrate concentrations at which the velocity of the enzyme reaction is one-half of its maximum velocity (Vmax). The

Km can also be termed the apparent Km, or the reciprocal of the apparent enzyme-substrate affinity (Hochachka and Somero, 1973), to distinguish it from the K_s, the enzyme substrate dissociation constant (Dixon and Webb, 1964).

C. Protein determinations.

The protein content of the tissue homogenates (soluble protein) was determined with Folin-Ciocalteu reagent by the method of Lowry et al. (1951). Absorbance was measured at 600 nm. Bovine serum albumin was used as a standard.

III. LIPID SYNTHESIS IN VIVO

A. Experimental protocol and blood samples.

The incorporation of labeled acetate, glucose, and tritiated water into lipids was used to measure lipid synthesis in vivo. Eels were weighed and placed into a separate tank containing approximately 35 liters of 15°C water for 12 to 48 hours prior to the experiment. Food was given to those eels which had previously been feeding. Animals were injected intraperitoneally at two to three points with either 0.5-2.0 µCi 1-^{14}C-acetate, 0.05-0.1 µCi 6-^{14}C-glucose, or 0.05-0.2 mCi $^{3}H_2O$ per g eel. In one experiment, eels were injected intramuscularly in the epaxial muscle beside the dorsal fin above the anus.

The injected eels were removed from the tank after the appropriate time period (see Results), weighed, then decapitated just anterior to the heart. Blood was collected from the severed conus arteriosus in heparinized 10 ml beakers, then centrifuged in microcentrifuge tubes (Eppendorf) for 10 min at 3/4 speed in an IEC clinical centrifuge.

The plasma was removed and duplicate 20 µl aliquots were counted for radioactivity, and the remaining plasma used for lipid or water determinations. The water content of eel plasma, determined gravimetrically, was 90.6 ± 0.4 (mean±SEM, n=6).

B. Lipid determination

Tissues were excised, weighed, and placed in polypropylene centrifuge tubes, and 19-79 volumes of chloroform-methanol (2:1 v/v) added. The tissues were homogenized with a Polytron PCU-2 tissue homogenizer (Brinkmann), centrifuged for 10 minutes at 10,000 x g, and then poured into 15 or 40 ml ground-glass topped centrifuge tubes through glass-fiber filter paper (Whatman GF/A). The pellet was resuspended in chloroform-methanol, vortexed for 1 min, re-centrifuged, and the supernatant poured through the filter. Small amounts of solvent were then used to wash the filters. In some experiments, the remaining pellet (lipid-free precipitate) was saved for determination of lipid-free dry weight and label incorporation.

The chloroform-methanol extracts were washed with 0.04% $CaCl_2$ (0.20-0.24 volumes), and then three times with prepared upper phase solvents (chloroform-methanol-0.4% $CaCl_2$, 6:96:94), as described by Folch et al. (1957). This procedure eliminated hydrophilic contaminants, and the last wash was substantially free of radioactivity. In the experiments in which labeled acetate was used as a lipid precursor, approximately 10 mg "cold" acetate was added to the third wash.

The lipid extracts were evaporated almost to dryness under nitrogen at 35°C, and made up to 3.0 or 5.0 ml volume with chloroform-methanol (approx. 4:1 v/v). The graduations on the tubes containing the lipid extract were checked by pipetting fixed volumes into the tubes. After

determining lipid weight gravimetrically, aliquots of lipid were used for the procedures discussed below.

C. Saponification.

Aliquots of up to 40 mg of lipid were transferred to 15 ml screw-topped tubes (teflon liners in caps) and the solvent evaporated under nitrogen. After the addition of 2.5 ml of 95-99% ethanol and 0.25 ml 6N KOH, the lipid mixture was saponified at 85°C for 2 hr. Ethanol and water (approx. 1:1 v/v) were then added to bring the final volume to 4.5 ml. Completeness of saponification was checked qualitatively by TLC and quantitatively by scintillation counting.

Non-saponifiable lipids were extracted with two to three 5 ml portions of petroleum ether (b.p. 30-60°C). Following acidification of the lipid mixture with approximately 0.35 ml 5N HCl, the fatty acids (saponifiables) were extracted in the same manner. Initially, both non-saponifiable (NS) and saponifiable (SAP) fractions were backwashed with equal volumes of dilute NaOH or HCl, respectively, but this was found to be unnecessary, except, as in a few experiments, when whole tissues rather than lipid extracts were saponified. Both the NS and SAP fractions were then placed in scintillation vials, evaporated, and counted for radioactivity. Alquots of these fractions were sometimes removed for weight determination and/or thin-layer chromatography. The aqueous fraction remaining after petroleum ether extraction (primarily lipid glycerol) was neutralized and duplicate 1.0 ml aliquots counted for radioactivity.

D. Thin layer chromatography (TLC) and lipid class determination.

In some experiments, the amount of isotope incorporated into the

different lipid classes and the weight percentage of each class was determined after separation by TLC. Aliquots of the lipid extract were placed in a tube and the solvent evaporated. Acetone precipitation (Kates, 1972, p. 394) was used to separate the polar lipids from the neutral lipids. Since this technique does not completely separate the polar from neutral lipids, both fractions were further separated with both polar and neutral solvent systems. The lipid fractions were then reduced in volume and spotted on thin layer plates or flexible supports with a stationary phase of silica gel G (250 μ) and developed in the following solvent systems: neutral lipids; ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2), followed by ethyl ether-hexane (6:94) (Freeman and West, 1966); polar lipids; chloroform-methanol-water (65:25:4) (Lepage, 1964).

Lipid classes were identified from the R_f values of lipid standards (Sigma), run concurrently with the eel lipid samples. The separated lipid classes were visualized with iodine vapor or, occasionally, with 2',7'-dichlorofluorescein (neutral lipids only) as described by Kates (1972, p.436). After gentle heating to remove the I_2, the detected lipids were removed by scraping the silica gel or cutting the flexible backing plus silica gel and placed in scintillation vials for the estimation of radioactivity. Silica gel did not interfere with scintillation counting, and recovery of labeled lipids from the chromatograms was 95-102%.

The spectrophotometric procedure of Amenta (1964) was used to determine the amount of lipid in each class separated by TLC. Silica gel containing lipid separated by TLC was incubated with an acid dichromate solution and the reduction in absorbance, proportional to

the amount of lipid present, was read at 350 nm. Triolein and cholesterol (Sigma) were used as standards.

IV. FATTY ACID SYNTHESIS IN TISSUE HOMOGENATES

The liver (or intestine) from worm-fed eels was excised, weighed, and homogenized in 3 volumes of dilute phosphate buffer (5 mM, pH 7.2) containing 250 mM sucrose and 1 mM dithiothreitol. After homogenization and centrifugation, as previously described (p.15), the pellet containing cell debris and mitochondria was discarded, and the 39000 x g supernatant centrifuged at 105,000 x g at 4°C for 1 hr in a Beckman LS-50 preparative ultracentrifuge. The microsomal-free supernatant from this procedure was added to the assay mixture within 30 minutes.

The assay mixtures for the incorporation of labeled substrates into fatty acids in the high-speed (microsomal-free) supernatants are, with slight modification, those used by Shrago et al. (1969), and contained the following components (final concentrations) in a total volume of 1.0 ml:

1,5-^{14}C-Citrate incorporation (citrate cleavage enzyme). 50 mM glycylglycine buffer, pH 7.5, 10mM ATP (Mg salt), 0.1 mM CoA, 0.3 mM NADPH, 25 mM $NaHCO_3$, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM dithiothreitol, 15 mM citrate, 1.5 µCi 1,5-^{14}C-citrate, and 0.4 ml high speed supernatant.

1-^{14}C-Acetate incorporation (acetyl CoA synthetase). Identical to citrate incorporation (above), except that labeled citrate was omitted, and 10 mM acetate and 2.0 µCi 1-^{14}C-acetate were added. Unlabeled citrate (15 mM) was present to stimulate acetyl CoA carboxylase (Shrago et al., 1969).

1-^{14}C-Acetyl CoA incorporation (acetyl CoA carboxylase). Identical to citrate incorporation, except that CoA and labeled citrate were

omitted, and 0.2 mM acetyl CoA and 0.6 μCi 1-^{14}C-acetyl CoA added. Unlabeled citrate (15 mM) was present.

1-^{14}C-Acetyl CoA + malonyl CoA incorporation (fatty acid synthetase). 50 mM potassium phosphate buffer, pH 7.0, 0.3 mM NADPH, 0.1 mM acetyl CoA, 0.6 μCi 1-^{14}C-acetyl CoA, 0.2 mM malonyl CoA, and 0.4 ml high speed supernatant.

The incubation media for citrate, acetate, and acetyl CoA incorporation also contained 1 mM phospho(enol)pyruvate and excess pyruvate kinase (rabbit muscle) to regenerate ATP, and 1 mM isocitrate to regenerate NADPH, and maintain a high citrate concentration.

The exact amount of acetyl CoA added to the assay mixtures was measured at 260 nm using a molar extinction coefficient of 16,400 (Dawson et al., 1969).

In each experiment, tubes containing the complete assay mixture, but without homogenate, were treated similarly to tubes containing both homogenate and substrate.

The assay was initiated by the addition of substrate after the homogenate had been "activated" (Sullivan et al., 1972) for 10 min in the assay media without substrate. The mixtures were incubated in screw-capped tubes for 90 min at 15°C in a shaking water bath.

The reactions were terminated by the addition of 2.0 ml 5 N KOH in 50% ethanol, and the mixture saponified at 85°C for 3 hr. Water was added to bring the volume to 2.6 ml, and 5 N HCl (2.4 ml) added to bring the pH to approximately 1.0. The fatty acids in the acidified digests were extracted with three 5 ml portions of petroleum ether (b.p. 30-60°C). The petroleum ether extracts were washed twice with water or dilute acetic acid, then added to scintillation vials where the ether was

removed under reduced pressure, scintillation fluid added, and the labeled fatty acids counted.

V. FATTY ACID SYNTHESIS IN LIVER SLICES

Eels used in these experiments were either fed to satiation with worms or starved for periods up to two years. The eels were killed by decapitation and the liver (or other tissues) immediately removed and weighed. The liver was sliced using a Stadie-Riggs Tissue Slicer (A.H. Thomas, Phila.) and the slices placed in cold, oxygenated fish saline, buffered to pH7.55 with HEPES (5 mM, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid). The composition of the fish saline (after Lin et al., 1977c) was as follows (grams/liter): NaCl, 7.25; KCl, 0.38; $CaCl_2 \cdot 2H_2O$, 0.23; $NaH_2PO_4 \cdot H_2O$, 0.41; $MgSO_4 \cdot 7H_2O$, 0.23; and $NaHCO_3$, 1.00.

Slices from each fish liver were randomly placed in three or four 25 ml Erylenmeyer flasks containing different substrates. Each flask contained approx. 150 mg of tissue. Because of the limited amount of tissue available, duplicate flasks were not routinely used (see p. 27 and Appendix 1), and generally, 3 animals, 4 conditions each, and a total of 12 flasks were utilized for each experiment. The liver slices were incubated in 3 ml of buffered saline to which 1% bovine serum albumin (Sigma A7511, essentially fatty acid free) had been added. The flasks were flushed with 95% O_2/5% CO_2, stoppered tightly, and the tissues incubated for 2 hr at 15°C in a shaking water bath. Preliminary experiments established that there was a linear increase in fatty acid synthesis (^3H_2O incorporation) for at least 3 hr.

After incubation, the tissues were removed immediately, except in those experiments in which CO_2 production was determined (see p. 26) and rinsed in three changes of fish saline, blotted, and weighed to the

nearest mg on a Roller-Smith balance. The slices were then transferred to 40 ml polypropylene centrifuge tubes, and the lipid extracted and saponified as described (p. 19). No labeled lipid was detected in the incubation media.

The various substrates added to the flasks are indicated in the Results section. In general, all cold substrates were added at a final concentration of 5 mM (15 μmoles/flask), while each flask contained 1 to 3 μCuries of ^{14}C-labeled substrate or 2 mCi of $^{3}H_2O$. The exact amount of isotope added was determined for each flask. The rates of lipid synthesis, in disintegrations per minute (dpm), were converted to corresponding nmole values using the specific activity of the substrate in the incubation flask. Insulin (bovine), at final concentrations up to 100 mU/ml, had no effect on fatty acid synthesis (see Results, p. 47), and was not routinely added to the flasks.

Acetoacetate was used as a substrate in one experiment. Ethyl-3-^{14}C-acetoacetate was converted to 3-^{14}C-acetoacetate, sodium salt, by hydrolysis for 4 hr at 40°C with an excess of NaOH as described by Edmond (1974). The sodium salt was neutralized with HCl and freeze-dried to reduce the volume.

VI. METABOLITES

A. Acetate.

Acetate was determined in liver and blood samples. Weighed tissues were added to 4 volumes of 6% (w/v) perchloric acid ($HClO_4$) at 0°C and homogenized with a Polytron tissue homogenizer. The precipitate was removed by centrifugation and re-extracted with 2 volumes of $HClO_4$. The combined supernatants were neutralized with 3 M $KHCO_3$, and after

several hours at 0°C, the precipitated $KClO_4$ was removed by centrifugation. The neutralized $HClO_4$ extracts were mixed with internal standards and measured by the method of Ackman (1972) on a Hewlett Packard 8720A Gas Chromatograph equipped with a model 3380 integrator. In addition to acetate, the internal standard also contained propionic, isobutyric, and butyric acids, none of which were detected in the eel tissue extracts. The GLC assays were performed in the NRC laboratory of Dr. Van den Berg by Mr. Duncan Wall.

Acetate was also determined by the spectrophotometric procedure of Knowles et al. (1974). This method of acetate estimation was complicated in some samples by spurious increases in absorbancy and non-linear responses, problems mentioned by Knowles et al. (1974) in connection with liver extracts.

B. Glycogen and glucose.

The procedures of Renaud and Moon (1980a) were used to determine these metabolites in tissues and plasma. Tissues were hydrolyzed with 30% KOH (w/v), glycogen extracted by alcoholic precipitation, and then hydrolyzed to glucose with 0.4N H_2SO_4 (Good et al., 1933). Glucose from the acid hydrolysate or from eel plasma was determined by the glucose oxidase-peroxidase method (Sigma bulletin 510) and the pink color formed after H_2SO_4 addition was measured at 540 nm.

VII. OXIDATION OF ^{14}C-OLEATE TO $^{14}CO_2$

A. Preparation.

Oleic acid (1-^{14}C) was converted to potassium oleate and bound to albumin by the procedure of Friedberg et al. (1960). Oleic acid (26 µCi)

was saponified in ethanolic KOH. The ethanol was evaporated and 0.63 ml fish saline, pH 7.8, containing 8% bovine serum albumin (fatty acid free) was added and gently stirred for 1 hr. More than 95% of the initial oleic acid radioactivity was recovered in the aqueous albumin solution.

Liver slices were prepared and incubated as described previously, except that CoA (0.5 mg) was added to the incubation medium (Raulin and Grundt, 1980). Approximately 2 µCi of 1-^{14}C-oleate (approx. 36 nmoles) was added to each flask. The exact amount of oleate added was determined for each flask. Flasks with oleate but no tissue were run as blanks.

B. CO_2 collection.

After 2 hr incubation the reaction was terminated by the addition of 0.3 ml 2N H_2SO_4. The flasks remained in the shaking water bath for an additional 2 hr while the $^{14}CO_2$ evolved was collected in 0.5 ml 1N hyamine hydroxide (NEN). The well containing the hyamine was placed in a scintillation vial and the $^{14}CO_2$ counted in Econofluor (NEN).

VIII. SCINTILLATION COUNTING

Non-aqueous samples were counted using 10 ml Econofluor (NEN) as the scintillant. Econofluor was added after all solvent had been removed from the sample under reduced pressure. Aqueous samples were counted with Aquasol-2 (NEN). The amount of Aquasol-2 added was routinely 10 ml, but in some experiments, this volume was increased to maintain a scintillant:sample ratio of at least 10:1 (v/v).

Prior to scintillation counting, lipid-free dry precipitates had to be dissolved. After rehydrating the lipid-free dry sample (20-50 mg) with 150 µl of water, 1.25 ml NCS tissue solubilizer (Amersham) was added

and the samples incubated at 55°C until dissolved; 15 ml Aquasol-2 and 25 µl glacial acetic acid were then added, and the vials placed in the dark for 24 hr prior to counting.

Labeled samples were counted for radioactivity on a LKB (Wallac) model 1215 Rackbeta liquid scintillation counter with programmed quench correction using an external standards ratio. Quench calibration was programmed using the "Hat-trick" method (LKB manual) with carbon tetrachloride as the quenching agent. The counting efficiency was 38-52% for ^{3}H and 80-90% for ^{14}C.

IX. CHEMICALS

All substrates, coenzymes, etc. were purchased from Sigma Chemical Co., St. Louis, Mo. or Boehringer-Mannheim, St. Laurent, Que. Inorganic reagents were purchased from local suppliers and were of the highest possible purity. All radioisotopes, scintillants, and scintillation vials were purchased from NEN Canada Ltd.

X. SAMPLE VARIABILITY AND STATISTICS

All results are expressed as mean±SEM. Most experimental preparations were routinely assayed in duplicate. An exception, due to limitations of tissue, was the in vitro experiments of lipid synthesis in liver slices.

The variability of in vitro lipid synthesis measurement and of saponification was determined by performing identical incubations in triplicate from three fishes (see Appendix 1). The SEM of the rate of lipid synthesis represented less than 2% of the mean of the triplicate incubations, and the SEM of the fraction % of fatty acids (saponifiables) recovered during saponification was less than 0.5% of the mean (Appendix 1).

Data were analyzed on a Wang Programmable Calculator (Wang Laboratories, Tewksbury, Mass.) with the linear regression program or the programs of t-statistics for paired or unpaired data. Differences between means were considered significant if $p<0.05$.

The data were assumed to be normally distributed, although, due to the small number of eels used for many of the experiments, comparison with expected chi-square distribution and other tests for normal distribution were not possible. When the variances were approximately equal, and not obviously related to the mean, t-tests (paired or unpaired) were used to compare the means between treatments. When the variances were unequal, or, in paired experiments, when they were related to the mean values, the data were logrithmically transformed (Steel and Torrie, 1960, p. 157) and t-tests then performed to compare the means.

RESULTS

I. TISSUE SITES OF LIPID SYNTHESIS

A. Labeled precursor experiments.

An initial estimate of the importance of each tissue in lipogenesis was made by measuring the rates of labeled acetate incorporation into tissue lipids (Table 2). The intestine had the highest rate of acetate incorporation, while other visceral tissues plus the brain and gills showed substantial incorporation. The muscle mass of the eel incorporated acetate very slowly, particularly white muscle, which comprises the largest weight % of any tissue. Whole muscle sections of the eel ("whole side"), which contained skin and subcutaneous fat in addition to red and white muscle, had only small amounts of label incorporation into lipids as well (Table 2).

It was considered possible that precursor availability could have restricted label incorporation into muscle lipids by the combination of peritoneal injection sites and low blood flow to the posterior tissues. However, when ^{14}C-acetate was injected directly into the epaxial muscle mass (whole side #2 sample of the 12 hr fish, Table 2), no major increase in label incorporation was observed, either compared to the whole side #1 sample (distal to the injection site) or when compared in relative terms to the visceral tissues. Therefore, the peritoneal site of injection, and, by inference, restricted label availability, were not responsible for the low incorporation rates observed in red and white muscle tissues.

The results presented in Table 2 were confirmed in subsequent

TABLE 2

In vivo incorporation of 1-^{14}C-acetate into the lipids of eel tissues.

Tissue	Weight %	Lipid Radioactivity (dpm/mg wet weight of tissue) 12 hr fish i.m.	12 hr fish i.p.	24 hr fish i.p.	120 hr fish i.p.	120 hr fish i.p.
Intestine	0.69 ± 0.06[1]	2285	719	202	1119	491
Brain	0.04 ± 0.01	1092	511	247	559	445
Gills	0.57 ± 0.06	1093	285	261	175	347
Liver	1.02 ± 0.06	628	252	453	373	303
Kidney	0.25 ± 0.07	657	155	158	184	333
Stomach	0.53 ± 0.05	254	123	84	295	690
Visceral fat	0.10	-	-	64	-	-
Spleen	0.22 ± 0.04	386	53	66	48	118
Skin	ca. 5.0	320	34	56	63	77
Heart	0.10 ± 0.01	98	29	99	40	60
Red muscle	1.19 ± 0.12	89	24	22	58	44
Whole side 1	89.8 ± 0.4	57	11	28	27	31
Whole side 2		65[2]	14	-	24	-
Blood	3.0[3]	73	10	40	37	66
White muscle	ca. 80.0	14	4	6	9	9

Experiments were conducted from March to May 1978 on 5 eels fed beef liver. Each vertical column represents one fish. Eels weighing 114.8 ± 5.7g (mean ± SEM) were injected i.p. or i.m. with 2 μCi/g 1-^{14}C-acetate (specific activity 54.6 mCi/mmole) and killed at the stated time (12-120 hr). [1]Mean percent of total body weight ± SEM. [2]Site of injection. [3]I. Cornish, pers. comm., determined by hematocrit dilution.

experiments using worm-fed eels killed 24 hr after injection (Table 3). The overall rates are lower than in Table 2 since only incorporation into the fatty acid fraction of the lipid extract is presented, *and* less labeled acetate was injected into each eel.

The values presented for each tissue in the in vivo experiments of Tables 2 and 3 may indicate not only de novo lipid synthesis, but also transport of lipids from sites of synthesis to those of storage or utilization. To determine whether the label incorporated into various tissues actually represented de novo synthesis in situ or whether it was the result of transport of lipids from other sites, the rate of fatty acid synthesis from acetate was measured in isolated slices of liver, intestine, gill, skin, and red muscle in vitro (Table 3). It is apparent that the liver had the greatest capacity for fatty acid synthesis, both in terms of total capacity (weight %) and per 100 mg tissue. Red muscle was the only tissue examined that had essentially no ability to synthesize lipids from acetate.

Based on these data (Tables 2,3), label incorporation in the liver, intestine, gills, and skin probably represents fatty acid synthesis in situ, while label incorporation into muscle lipids undoubtedly reflects transport from other sites.

Acetate does not represent the only possible precursor of tissue fatty acids. Labeled glucose may be incorporated into fatty acids and other lipid fractions in vivo by entirely different pathways than acetate (see Introduction). Some of these pathways may be more important in muscle compared to visceral tissues. However, when 6-^{14}C-glucose was injected into eels (Table 3), the muscle fatty acids incorporated virtually no label in 24 hr (less than 1 dpm/mg), while both

TABLE 3

Incorporation of 1-^{14}C-acetate and 6-^{14}C-glucose into fatty acids (FA) of selected eel tissues.

Tissue	Weight %	In vivo FA Synthesis ^{14}C-acetate (n=1 or 7)	In vivo FA Synthesis ^{14}C-glucose (n=5)	In vitro FA Synthesis ^{14}C-acetate (n=3)
Liver	1.17 ± 0.07[1]	292 ± 54	18 ± 4	31.3 ± 7.1
Intestine	0.79 ± 0.06	437 ± 106	16 ± 4	8.2 ± 0.6
Visceral fat	0.10	64		
Gill	0.57 ± 0.06	261		4.5 ± 0.4
Whole side	89.8 ± 0.4	13 ± 2	<1	
Skin	ca. 5.0	56		4.2 ± 0.4
Red muscle	1.86 ± 0.17	17 ± 3		0.06 ± 0.01
White muscle	ca. 80.0	4 ± 1		

In vivo fatty acid synthesis is expressed as dpm in fatty acids/mg wet wt of tissue (mean ± SEM). 1-^{14}C-acetate (1-2 μCi/g) or 6-^{14}C-glucose (0.1 μCi/g) were injected i.p. and the eels killed 24 hr later. The acetate experiments were performed in 1978 (n=1, from Table 2); 1979 (n=3); 1980 (n=3). Glucose experiments are from 1979 (n=5). Visceral fat, gill, and skin in vivo data represent the fatty acid fraction from the 24 hr eel of Table 2. Eels were fed beef liver (1978) or worms (1979, 1980).

In vitro fatty acid synthesis is expressed as nmoles 1-^{14}C-acetate incorporated into fatty acids/100 mg wet wt/3 hr at 15 C (mean ± SEM). The eels were fed beef liver.

[1]Mean percent of total body weight.

liver and intestine incorporated, in relative terms, substantial amounts (18 and 16 dpm/mg, respectively). These results (Table 3) confirm those of the acetate incorporation experiments (Tables 2,3), and suggest that muscle fatty acid synthesis from these precursors is negligible.

With saponification and isolation of fatty acid and glycerol fractions in the acetate experiments, 30-40% of the lipid label of all tissues was found in the glycerol moiety (fraction % is 30-40%). This is compared to the larger and more varied glycerol fraction % in the glucose experiments, where liver lipids contained 45% of their label in glycerol; intestine, 60%; and whole eel side, 80%. Further characterization of the newly synthesized lipids is given in Appendix 3.

B. NADPH production and related enzyme activities.

The comparative ability of selected eel tissues to produce fatty acids was further evaluated by measuring the activity of cytoplasmic NADP-linked enzymes. The activity of these enzymes is positively correlated with fatty acid synthesis in several species and tissues (Romsos and Leveille, 1974).

The pattern of enzyme activity differs in each tissue as follows (Table 4): In the liver, active IDH and pentose phosphate pathway enzymes (G6PDH, 6PGDH) were found, but there were only traces of malic enzyme activity (only two of ten 1977 animals had detectable activity, neither exceeding 0.15 μmoles/min/g). In visceral fat, G6PDH and 6PGDH were at least four times as active as IDH or malic enzyme. With the exception of ME, all enzymes were less active than those in the liver. In the intestine, all four NADP-linked enzyme activities were nearly equivalent. In red muscle, IDH and malic enzymes were active, but there was little G6PDH or 6PGDH activity, while in white muscle, the

TABLE 4

Activity of cytoplasmic NADPH producing enzymes and malate dehydrogenase in different eel tissues[1]

Tissue	(n)	IDH	G6PDH	6PGDH	ME	MDH	TOTAL NADPH[2]
Liver	(10)	6.45 ± 0.28[3]	0.86 ± 0.12	3.53 ± 0.28	tr	416.5 ± 26.5	10.84
Visceral fat	(5)	0.12 ± 0.03	0.50 ± 0.09	0.44 ± 0.07	0.07 ± 0.02	14.3 ± 3.3	1.13
Intestine	(8)	0.98 ± 0.07	1.19 ± 0.03	1.09 ± 0.06	0.64 ± 0.06	67.2 ± 5.3	3.90
Red muscle	(8)	3.13 ± 0.24	0.06 ± 0.01	0.16 ± 0.01	0.63 ± 0.05	138.2 ± 8.0	3.98
White muscle	(10)	0.45 ± 0.03	tr	0.05 ± 0.01	0.36 ± 0.03	34.2 ± 3.3	0.86

Enzyme activity is expressed as μmoles of NADPH (or NAD) produced/min/g wet weight of tissue. [1]Eels were captured in 1977. [2](G6PDH x 2) + IDH + ME. [3]Mean ± SEM. The number of eels is given in parenthesis. Abreviations: IDH, isocitrate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; ME, malic enzyme; MDH, NAD-malate dehydrogenase; tr., trace of activity (less than 0.03 μmoles/min/g). Since no significant differences (P>0.05) in enzyme activities were found in any tissue when comparing beef liver-fed and 2 month fasted eels, the data from both groups were combined.

activity of all four NADP-dependent enzymes was low. NAD-malate dehydrogenase was very active in all tissues. In the liver, aconitase was found only in the cytoplasm (as was ATP-citrate lyase), confirming the report of Moon and Ouellet (1979). The intestine also contained both aconitase (0.11±0.03 μmoles/min/g, n=4, mostly mitochondrial) and ATP-citrate lyase (0.22±0.04 μmoles/min/g, n=4), while the red muscle contained aconitase (0.28±0.01 μmoles/min/g, n=3, mostly mitochondrial) but no ATP-citrate lyase.

The total potential NADPH production through the pentose phosphate pathway ((G6PDH activity) x 2) plus IDH and malic enzyme (Table 4, extreme right) in the liver was more than double that of the intestine and red muscle, and seven to ten times greater than that of visceral fat or white muscle. This comparison is the same whether it is made per gram wet weight of tissue (as in Table 4) or per total tissue per animal, since the individual weights of the visceral fat, intestine, or red muscle are usually less than, or only slightly exceed (red muscle) the weight of the liver in eels of this size (see weight %, Tables 2,3). The extremely low NADP-linked enzyme activities in white muscle indicate that this tissue may be conveniently ignored in this comparison. The relatively high total potential NADPH production of red muscle (Table 4) is in contrast to the low in vivo and in vitro incorporation of acetate into fatty acids (Tables 2,3). However, nearly all of the potential NADPH production is from one enzyme, IDH (Table 4). This observation suggests a role unrelated to fatty acid synthesis for this enzyme in red muscle. In other tissues, the relative NADPH production substantially concurs with the magnitude of label incorporation (Tables 2,3).

II. HEPATIC LIPOGENESIS: REDUCING EQUIVALENTS AND CARBON SOURCES

Both the liver and intestine exhibited consistently high rates of label incorporation into lipids and active NADP-linked enzyme activities compared to other eel tissues (Tables 2-4). Based on these results, and the availability of considerable related information on hepatic metabolism in eels (Moon and Ouellet, 1979; Renaud and Moon, 1980a,b), the liver was examined in greater detail specifically to establish the source(s) of reducing equivalents and carbon for fatty acid synthesis.

A. The effect of diet on hepatic lipogenic enzyme activities and related parameters.

1977 eels. There were no statistically significant differences in enzyme activities per gram liver between eels fed beef liver and those fasted two months (Table 5). However, the total potential NADPH production was higher in the fed group due to an increased liver weight. Neither group exhibited quantifiable ATP-citrate lyase activity.

In extra-hepatic tissues, as well, no significant differences in enzyme activities were found between the two groups (Table 4). The data presented in Table 4 are thus the combined values from both fed and 2-month fasted animals.

Despite the small sample size (n=3), freshly caught fish showed significantly greater G6PDH and aconitase activities and greater total potential NADPH production per liver compared with either beef liver fed or fasted eels (Table 5).

1978 eels. Fish fed earthworms showed much greater liver G6PDH activity and liver weight than those fish fasted for 4 to 6 months (Table 5). These two factors resulted in a nearly two-fold difference in potential NADPH production per liver.

Table 5 (*footnotes*). [1]Enzyme activity is expressed as μmoles of NADPH (or NAD) produced/min/g wet weight of liver. [2]Mean ± SEM. [3]Within each year or dietary comparison, the superscript "b" indicates a significantly different value from "a" ($p>0.05$). Absence of superscript letters indicates no significant differences ($p<0.05$) between means. Groups from different years were not compared statistically. [4]mg/g wet weight of liver. [5]% dry weight. [6]Includes skin, subcutaneous fat, red muscle, and white muscle. [7]Hepato-somatic index, Liver weight/Body weight x 100 (Liver weight per 100g eel weight). [8]HSI x ((G6PDH x 2) + IDH). Abbreviations: IDH, isocitrate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; ME, malic enzyme; CCE, ATP-citrate lyase; tr., trace (less than 0.03 μmoles/min/g).

TABLE 5 Effect of diet and fasting on lipogenic enzyme activities[1] and other parameters in eel liver

	1977			1978		1979
	Freshly caught	Fed (beef liver)	Fasted (2 months)	Fed (worms)	Fasted (4-6 months)	Freshly caught
n	3	5	5	7-10	7-10	6
IDH	6.90± 0.85[2]	6.50±0.33	6.40±0.50	6.23±0.71	6.70±0.36	-
G6PDH	1.79± 0.30[a 3]	0.74±0.12[b]	0.98±0.21[b]	2.46±0.69[a]	0.91±0.18[b]	-
6PGDH	3.19± 0.82	3.42±0.53	3.64±0.24	-	-	-
ME	tr	tr	tr	tr	tr	-
CGE	-	tr	tr	0.28±0.07	0.14±0.03	0.46±0.06
Aconitase	1.16± 0.06[a]	0.80±0.07[b]	0.71±0.12[b]	1.95±0.19	2.21±0.26	4.20±0.28
Soluble protein[4]	-	64.2 ±2.5 [a]	84.2 ±1.6[b]	67.5 ±3.0[a]	76.2 ±2.8[b]	-
% Lipid[5] :Liver	-	-	-	7.64±0.89[a]	12.63±1.37[b]	-
% Lipid[5] :Muscle[6]	-	-	-	10.03±1.12	14.06±3.04	-
Body wt	117 ±13.0	110 ±7.8	103 ±6.6	120.8 ±8.7	128.3 ±7.8	96 ±9.8
HSI[7]	1.19± 0.19	1.37±0.10	1.19±0.09	1.58±0.14[a]	1.06±0.02[b]	1.22±0.03
NADPH prod.[8] per liver	12.47	10.93	9.95	17.62	9.03	-

ATP-citrate lyase was more active in fed fish ($0.05<p<0.1$), although there was considerable variation between eels (Table 5). Aconitase activity was not affected by diet and was at least seven-fold more active than ATP-citrate lyase. The lipid content of the fasted eels was equal to or greater than that of fed eels, both in the liver and whole muscle sections, whether expressed as a percentage (Table 5) or as total lipid per liver of a 100 g eel (HSI x % lipid).

1979 eels. Freshly caught animals were used to determine whether the ratio of liver aconitase activity to ATP-citrate lyase activity differed in animals on a natural diet compared to those maintained in the lab. Aconitase activity was nearly 10-times greater than ATP-citrate lyase activity, confirming 1978 results (Table 5). Both aconitase and ATP-citrate lyase activities (1978) were two-fold higher in the freshly caught fish than in fish maintained in the laboratory (1978) (Table 5).

Although emphasis has been placed on comparisons between parameters of eels captured in the same year, several overall observations can be made from data in Table 5. First, IDH activity is not affected by diet, while G6PDH activity is. Secondly, fasting decreases the liver size. Finally, total potential NADPH production decreases in the order fed (worms)>freshly caught>fed (beef liver)>fasted.

B. Kinetic parameters.

The competition for coenzyme, substrate and the degree of product inhibition by NADPH may influence cellular activities of the various NADPH-producing enzymes as significantly as their maximal activities. The apparent affinities of liver enzymes (and intestinal ME) for substrate, coenzyme and product are shown in Table 6. IDH has the greatest

TABLE 6

Michaelis constants of cytoplasmic NADP-dehydrogenases, ATP-citrate lyase, and aconitase from eel liver

	Km(NADP)	Ki(NADPH)	Km(substrate)[1]
IDH	2.3 ± 0.2	29.1 ± 8.4	6.0 ± 1.5
G6PDH	4.7 ± 0.6	17.2 ± 2.7	15.0 ± 2.6
6PGDH	4.6 ± 0.7	68.0 ± 6.3	17.1 ± 1.0
Malic enzyme[2]	4.5 ± 0.5	25.9 ± 1.2	170 ± 11
ATP-citrate lyase			3466 ± 1266
Aconitase			152 ± 7

Michaelis constants are expressed as μmoles/liter, mean ± SEM for 3-5 fish. [1]Substrates used: isocitrate for IDH, G6P for G6PDH, 6PG for 6PGDH, malate for malic enzyme, and citrate for ATP-citrate lyase and aconitase. [2]Malic enzyme from eel intestine.

apparent affinity for NADP, while the Ki(NADPH) values for all but 6PGDH are similar. Mitochondrial IDH has essentially identical kinetic values to the cytoplasmic enzyme (data not presented).

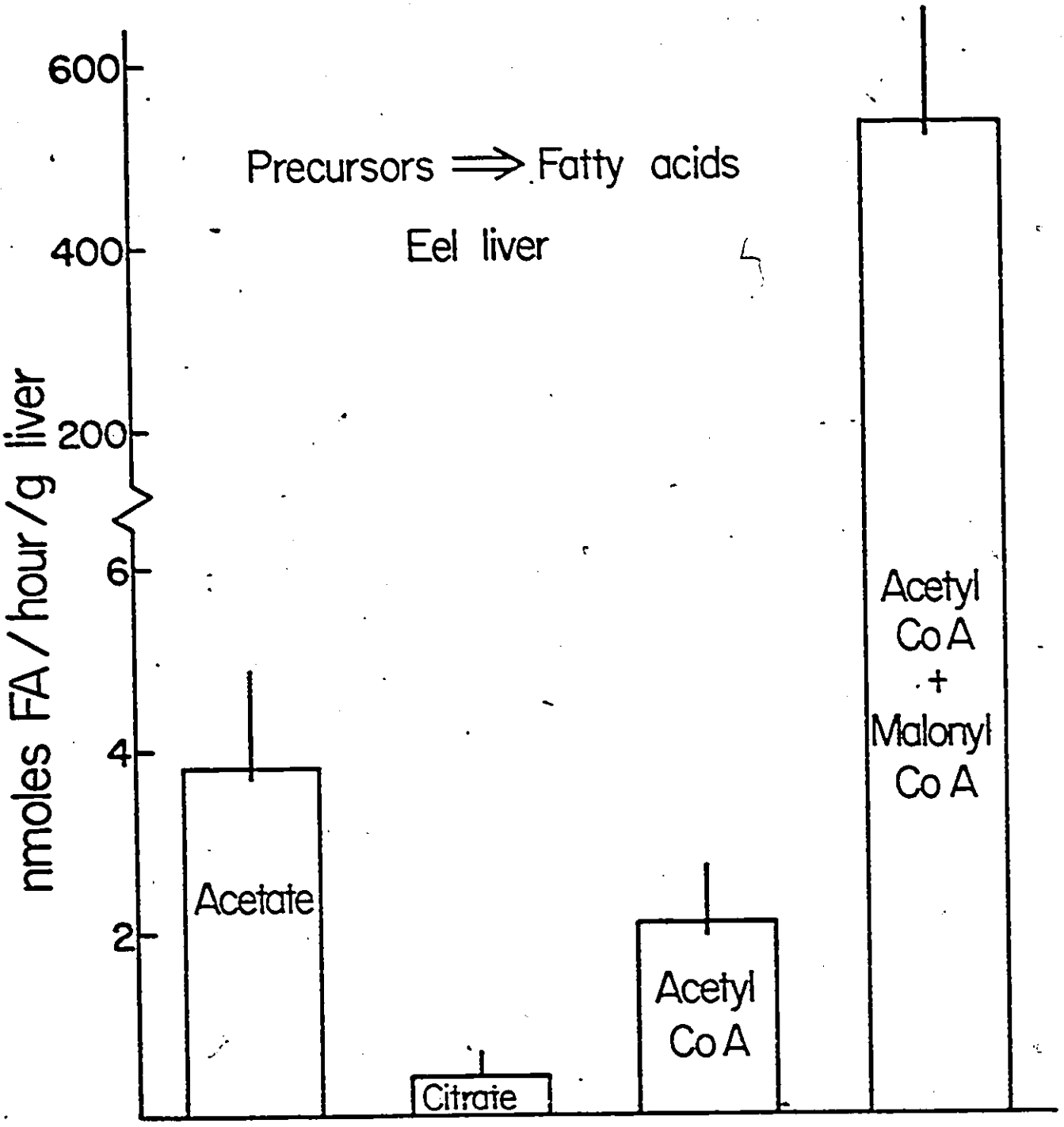
ATP-citrate lyase and aconitase both utilize cytoplasmic citrate. However, aconitase has a 20-fold greater apparent affinity for citrate (Table 6), and this plus a 10-fold greater activity (Table 5) should favor aconitase over ATP-citrate lyase in any competition for this common substrate in vitro; in vivo, flux may be controlled differently (see below).

C. Carbon sources for hepatic fatty acid synthesis: in vitro incorporation of labeled precursors.

The enzyme studies (Tables 5,6) indicated that ATP-citrate lyase may not be favored in competition with aconitase for the common substrate, citrate. Consequently, citrate, and other carbon sources requiring citrate cleavage to produce extra mitochondrial acetyl CoA, should be incorporated into fatty acids more slowly than those carbon sources, such as acetate which do not require citrate cleavage. To test this hypothesis, several types of in vitro label incorporation experiments were performed.

The rate of labeled acetate incorporation into fatty acids in high-speed supernatants was several-fold greater than that of citrate (Fig. 3). However, these rates were extremely low in comparison with acetate incorporation into liver slices (Table 3, see also Table 9). Supernatants containing microsomes exhibited the same low rates. There was no detectable in vitro incorporation of either labeled acetate or citrate into the lipids of a visceral fat supernatant (n=1), although in previous experiments (Table 3), acetate carbon was incorporated in vivo. The low rate of labeled acetyl CoA incorporation compared with that of ^{14}C-

Figure 3. The incorporation of ^{14}C-labeled precursors into fatty acids in liver homogenates. Fatty acid synthesis is expressed as nmoles ^{14}C-acetyl units incorporated into fatty acids/hr/g wet weight of liver. The following precursors were used: 1-^{14}C-acetate (10 mM, 2.0 µCi); 1,5-^{14}C-citrate (15 mM, 1.5 µCi); 1-^{14}C-acetyl CoA (0.2 mM, 0.6 µCi); and 1-^{14}C-acetyl CoA (0.1 mM, 0.6 µCi) plus "cold" malonyl CoA (0.2 mM). 1-^{14}C-acetate incorporation was significantly greater than 1,5-^{14}C-citrate incorporation ($p<0.05$), and 1-^{14}C-acetyl CoA plus malonyl CoA incorporation was significantly greater than that of 1-^{14}C-acetyl CoA alone ($p<0.001$).


Precursors ⟹ Fatty acids
Eel liver
nmoles FA / hour / g liver
600
400
200
6
4
2
Acetate
Citrate
Acetyl Co A
Acetyl Co A + Malonyl Co A

acetyl CoA plus malonyl CoA (Fig. 3), suggests that the conversion of acetyl CoA to malonyl CoA was severely rate-limiting in this supernatant preparation, and, therefore, no reliable estimate of the relative rates of citrate vs. acetate incorporation could be obtained. A homogenate of eel intestine (n=1) incorporated ^{14}C-acetyl CoA into lipids in the presence of malonyl CoA at about 30% (168 nmoles FA/hr/g) of the rate in liver homogenates. This proportion is similar to that observed in tissue slice experiments (Table 3).

The extremely low rates and inconclusive data obtained from the hepatic high-speed supernatants (Fig. 3) required that another preparation, liver slices, be used to assess the best in vitro conditions for lipogenesis and the relative contributions of different precursors.

It has been demonstrated that the incorporation of tritium from $^{3}H_2O$ (actually ^{3}HOH) into lipids is a reliable estimate of lipogenesis in vitro under a variety of conditions (Jungas, 1968; see Discussion). The effects of different substrates on the rates of $^{3}H_2O$ incorporation into fatty acids are shown in Tables 7 and 8. The great variation in rates of fatty acid synthesis between eels (range 24 to 492 nmoles $^{3}H_2O$ incorporated/100 mg/2 hr in fed eels) required that all comparisons be made with paired samples (slices from the same eel liver).

The addition of lactate (with glucose) to the incubation medium was required for maximal lipid synthesis in eel liver slices (Table 7), while acetate alone, or glucose with substrates other than lactate, did not increase the rate of lipogenesis substantially above the rate attained when no exogenous substrate was present. Of the substrates tested with glucose, however, the addition of acetoacetate gave the highest rates compared with glucose plus lactate.

TABLE 7

The effect of added substrates on fatty acid synthesis in liver slices from fed and fasted eels.

Expt. no.	(n)	Substrates + ^3H_2O						
		Glucose+ lactate	None	Acetate	Glucose+ acetate	Glucose+ acetoacetate	Glucose+ leucine	Glucose+ α-ketoisocaproate
Fed:								
1	7	167.5 ± 53.9	55.8 ± 16.3***					
2	3	106.7 ± 30.9		37.4 ± 9.3**	37.6 ± 7.7**			
3	3	276.1 ± 90.6				207.5 ± 111.7*	117.5 ± 39.5***	
Fasted:								
1	3	34.6 ± 10.0	17.5 ± 2.5*			18.6 ± 1.3*		22.8 ± 5.6**

Fatty acid synthesis is expressed as nmoles ^3H_2O incorporated into fatty acids/100 mg wet weight/2 hr at 15 C (mean ± SEM). The fasted eels were deprived of food for 4 months. The various additions to the incubation flasks were tested using paired experiments. All additions were compared with flasks which contained glucose+lactate+^3H_2O. Substrate concentrations were 5 mM (15 μmoles/flask). Significance of differences between means of paired experiments was tested on log transformed data with a paired t-test, * $p<0.1$; ** $p<0.05$; *** $p<0.01$.

TABLE 8

The effect of substrate addition on fatty acid synthesis in liver slices in the presence of glucose+lactate

		Substrates + ^3H_2O						
Expt. no.	(n)	Glucose+ lactate	Glucose+ lactate+ citrate	Glucose+ lactate+ aspartate	Glucose+ lactate+ alanine	Glucose+ lactate+ acetate	Glucose+ lactate+ acetoacetate	Glucose+ lactate+ α-ketoisocaproate
1,2	6	187.4 ± 69.9	208.5 ± 75.2**					
3	3	237.1 ± 127.5		222.3 ± 100.8	224.1 ± 99.2			
4,5	6	246.8 ± 55.7				231.0 ± 48.6		
6	3	300.8 ± 107.8					364.4 ± 136.0	291.8 ± 95.8

See legend to Table 7 for details, ** $p<0.02$.

Upon saponification of the lipid extracted from the liver slices, tritium label was found predominantly (66%) in the fatty acid fraction of the newly synthesized lipids (fed eels, Table 11). With the exception of glucose plus acetoacetate, slices incubated without glucose and lactate showed a lower fraction % of labeled fatty acids and a higher fraction % of labeled glycerol (Appendix 2) (fraction % is the precursor label in a lipid fraction x 100/precursor label in total lipid extract).

The addition of insulin to the incubation medium had no effect on rates of fatty acid synthesis (no insulin, 89.9±48.0; 100 mU/ml, 90.6±47.6 nmoles ^3H_2O incorporated into FA/100 mg/2 hr, n=3) or on the fraction % of fatty acids (no insulin, 54.3±15.0%; 100 mU/ml, 55.4±14.8%).

When the addition of glucose and lactate was tested against these two plus one additional substrate (Table 8), no increase in rates of ^3H_2O incorporation into fatty acids was observed when either aspartate, alanine, acetate, or α-ketoisocaproate was the additional substrate. The presence of acetoacetate increased fatty acid synthesis, but the increase was not significant statistically ($p>0.1$). Citrate addition, however, did result in a statistically significant increase in tritium incorporation (Table 8).

These results (Tables 7,8) demonstrate the importance of the presence of lactate for high rates of ^3H_2O incorporation into fatty acids. However, one cannot determine from these data whether lactate is providing carbon, reducing equivalents, or both.

From hepatic enzyme activities and kinetics (Tables 5,6), particularly of aconitase and ATP-citrate lyase, it was predicted that those precursors, such as citrate, lactate, glucose, and glucogenic amino acids, requiring citrate cleavage to generate extramitochondrial acetyl

CoA would be utilized as carbon sources for lipogenesis only sparingly, and much more slowly than those substrates not requiring citrate cleavage, such as acetate and acetoacetate. The experiments in Table 9 test this prediction directly.

These experiments (Table 9) compare the incorporation of tritium from ^3H_2O with the incorporation of labeled carbon from a variety of substrates in paired incubations. At least two labeled carbon precursors were used in each paired experiment. It was assumed, as an approximation (Clark et al., 1974; see Discussion), that the incorporation of one nanoatom of ^3H equals the incorporation of one nanoatom of carbon into fatty acids, hence one nmole ^3H_2O (actually ^3HOH) equals one acetyl unit as expressed in Table 9. The data (Table 9) clearly indicate that acetate and acetoacetate are incorporated into fatty acids to a significantly greater ($p<0.05$) extent than lactate, citrate, aspartate, or glucose, whether expressed as the actual rate in nmoles ^{14}C incorporated (in acetyl units) or as the ratio of $^{14}C_2/^3H_2O$ expressed as a percentage. If ^3H_2O incorporation is an accurate measure of the total rate of fatty acid synthesis, then acetate or acetoacetate, in the presence of glucose and lactate, account for approx. 70% of the carbon incorporated into fatty acids; lactate, even in the absence of acetate or acetoacetate, accounted for approx. 10% of the newly synthesized fatty acid carbon. Incorporation of ^{14}C into lipid glycerol was less than 10% of ^3H_2O incorporation for all substrates (lactate, acetate shown in Table 10B), with the exception of ^{14}C-aspartate where the ^{14}C-label was 67-76% of the tritium ($n=3$).

Thus the fatty acid results obtained from the paired label incorporation experiments (Table 9) support the hypothesis that citrate

TABLE 9

Incorporation of ^{14}C-labeled precursors into fatty acids in liver slices, and a comparison with $^{3}H_2O$ incorporation into fatty acids

Glucose+ lactate+ ^{14}C-precursor	(n)	$^{14}C_2$ ⟶ fatty acids[1]	$^{14}C_2/^{3}H_2O$ X 100[2]
1-^{14}C-acetoacetate	4	82.4 ± 46.7	72.5 ± 22.0
1-^{14}C-acetate	5	91.3 ± 31.2	65.8 ± 6.1
3-^{14}C-lactate	11	17.2 ± 6.7 *	10.0 ± 2.7 **
1,5-^{14}C-citrate	3	16.3 ± 11.9 *	8.2 ± 3.2 **
U-^{14}C-aspartate	3	0.7 ± 0.1 **	2.3 ± 0.4 **
6-^{14}C-glucose	3	0.1 ± 0.01**	0.4 ± 0.01**

[1]Fatty acid synthesis expressed as nmoles ^{14}C-acetyl units incorporated into fatty acids/100 mg wet wt/2 hr at 15 C (mean ± SEM). [2]Ratio of nmoles ^{14}C-acetyl units/nmoles $^{3}H_2O$ incorporated into fatty acids in paired experiments expressed as a percentage. It is assumed, as an approximation (Clark et al., 1974), that one nmole of acetyl unit represents one nmole of $^{3}H_2O$ (^{3}HOH) incorporated into fatty acids. Significance of differences between means was tested using t-tests to compare acetate or acetoacetate with all other precursors with lower rates or ratios, * $p<0.05$; ** $p<0.01$. The nmoles of $^{3}H_2O$ incorporated into fatty acids in the paired experiments are not shown but may be calculated by the formula, $100/(\%^{14}C_2/^{3}H_2O)$ x nmoles $^{14}C_2$ incorporated.

cleavage is not an important source of extramitochondrial acetyl CoA for lipogenesis, a prediction made from the enzyme activity and kinetic estimates (Tables 5,6).

III. LIPOGENESIS: EFFECTS OF FOOD-DEPRIVATION

A. In vitro measurements.

The effects of food-deprivation on hepatic fatty acid and lipid glycerol synthesis are illustrated in Fig. 4. Food deprivation resulted in a large, statistically significant ($p<0.05$) decrease in the synthesis of fatty acids by eel liver slices. After 4 months of fasting, only 23% of the fed rate of fatty acid synthesis was obtained, and after 9 months of starvation only 5%, which decreased to 1% in the 22 month starved eel. Labeled acetate and lactate incorporation into fatty acids showed a similar progressive decrease with starvation (Table 10A), although the relationship between ^{14}C and $^{3}H_2O$ incorporation, identical between fed and 9 month starved eels, changed considerably in the one 22 month starved animal examined (Table 10B).

The rate of $^{3}H_2O$ incorporation into lipid glycerol increased slightly after 4 months fasting, despite the large decrease in fatty acid synthesis (Fig. 4). Glycerol synthesis decreased only modestly with longer periods of food-deprivation. Labeled lactate and acetate incorporation into glycerol increased slightly in starved eels, both in actual rates (nmoles) and as a percentage of $^{3}H_2O$ incorporation. It has been shown that nmoles of $^{3}H_2O$ incorporated into glycerol are approximately equal to nmoles acetyl units incorporated (Jungas, 1968).

As expected from the above results, starvation decreased the percentage of label found in the fatty acid fraction upon saponification,

Figure 4. The effect of food-deprivation on fatty acid and lipid glycerol synthesis in eel liver slices. Synthesis is expressed as nmoles ^3H_2O incorporated into fatty acids or glycerol/100 mg wet weight/2 hr at 15°C (mean±SEM) in the presence of glucose (5 mM) and lactate (5 mM). Significance of differences between means comparing fed vs. starved eels was tested on log transformed data, * $p<0.05$; ** $p<0.001$.

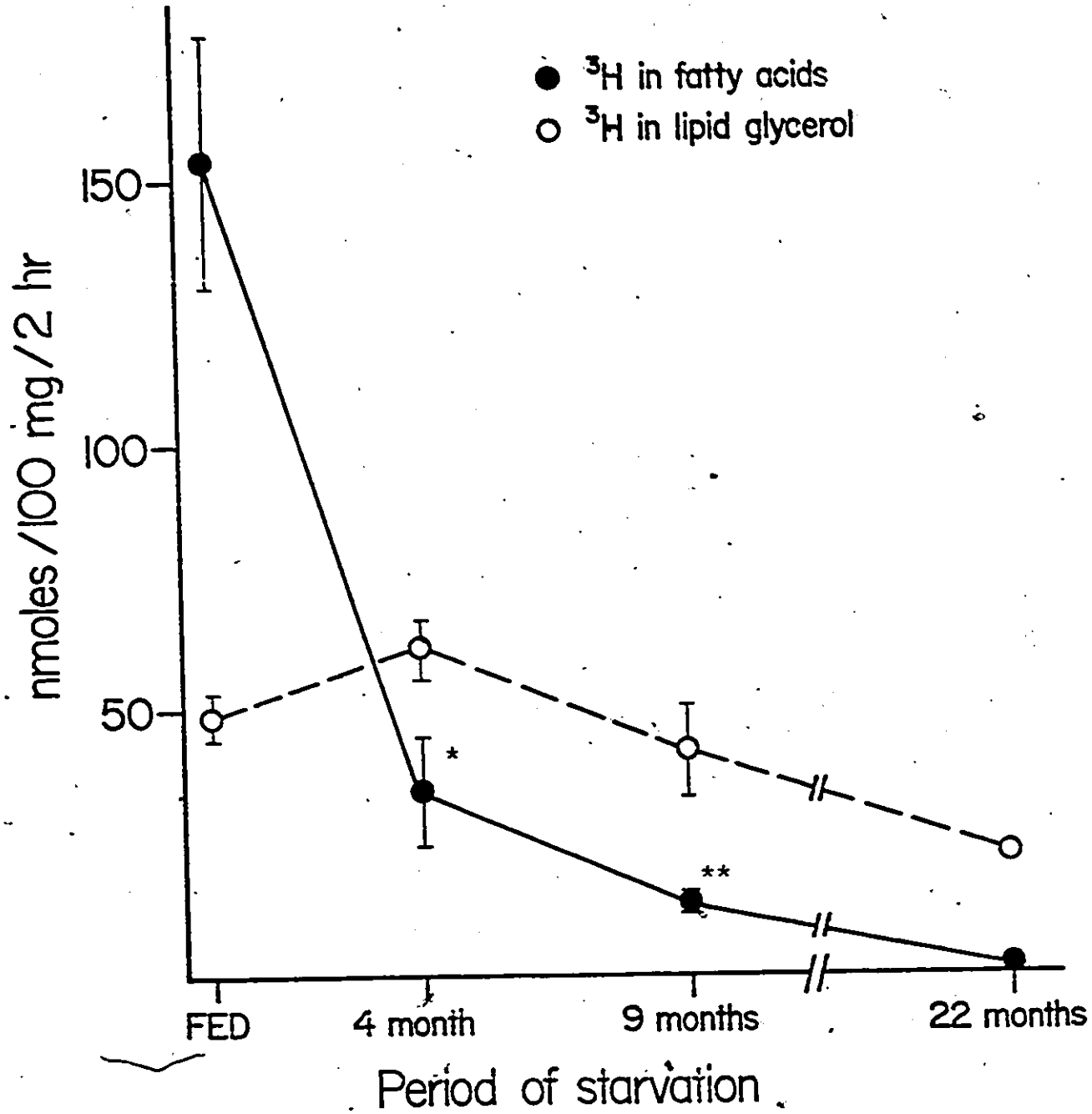

● ³H in fatty acids
○ ³H in lipid glycerol
150
100
50
nmoles /100 mg /2 hr
*
**
FED
4 month
9 months
22 months
Period of starvation

TABLE 10A

Effect of starvation on the synthesis of fatty acids (FA) and lipid glycerol from ^{14}C-acetate and ^{14}C-lactate in liver slices

	$^{14}C_2$ nmoles→fatty acids		$^{14}C_2$ nmoles→lipid glycerol	
Condition (n)	Acetate	Lactate	Acetate	Lactate
Fed (5,11)	91.3 ± 31.2	17.2 ± 6.7	0.4 ± 0.1	3.1 ± 0.4
Starved:				
9 months (3)	9.1 ± 3.5*	1.9 ± 1.0*	0.4 ± 0.1	4.0 ± 1.2
22 months (1)	3.4*	0.7*	0.6	2.8

Synthesis is expressed ad nmoles ^{14}C-precursor incorporated into FA or glycerol/100 mg wet weight/2 hr at 15° C (mean±SEM). All incubation flasks contained glucose (5mM), lactate (5mM) plus either ^{14}C-acetate (5mM, 2 μCi/flask) or ^{14}C-lactate (1 μCi/flask). Fed results are from Table 9. Significance of differences between means comparing fed vs. starved eels, *$p<0.05$.

TABLE 10B

Effect of starvation on the ratio (%)[1] of ^{14}C-precursor to $^{3}H_2O$ incorporated into fatty acids and lipid glycerol in liver slices.

		% $^{14}C_2/^{3}H_2O$ fatty acids		% $^{14}C_2/^{3}H_2O$ lipid glycerol	
Condition	(n)	Acetate	Lactate	Acetate	Lactate
Fed	(5,11)	65.8 ± 6.1	10.0 ± 2.7	1.5 ± 0.1	7.5 ± 0.9
Starved:					
9 months	(3,3)	66.8 ± 17.1	12.8 ± 5.7	1.0 ± 0.1	9.5 ± 1.7
22 months	(1,1)	230.9	44.3	2.8	12.4

[1]Ratio of nmoles ^{14}C-acetyl units/nmoles $^{3}H_2O$ incorporated into fatty acids or glycerol in paired experiments expressed as a percentage. It is assumed, as an approximation (Clark et al., 1974) that one nmole acetyl unit represents one nmole $^{3}H_2O$ (^{3}HOH) incorporated into fatty acids or glycerol. Rates of incorporation of ^{14}C-precursors into lipids are presented in Table 10A. Rates of $^{3}H_2O$ incorporation in fatty acids and glycerol are not shown but may be calculated as in Table 9.

and increased the percentage of labeled glycerol (Table 11). The magnitude of these changes for $^{3}H_2O$ and ^{14}C-lactate was similar but was less marked for labeled acetate. The fraction % of label incorporated into non-saponifiables generally decreased with starvation, but accounted for less than 5% of the labeled lipids under all conditions (Table 11).

B. In vivo measurements.

To establish that the effects of starvation on fatty acid synthesis in liver slices (Fig. 4, Tables 10A,B) were representative of the intact animal, $^{3}H_2O$ was injected into worm-fed and 9 month starved eels, and the tritium incorporated into hepatic lipids after 30 hours was determined. Synthesis of fatty acids by the liver was 30-fold greater in fed than in starved eels in vivo (Table 12), exceeding the 10-fold difference found in vitro (Fig. 4). Synthesis of non-saponifiable lipids was only 2-fold greater in fed eels (Table 12). These results confirm the previous results (Fig. 4, Table 10A) that showed lipid synthesis to be greatly diminished in starved eels.

Although $^{3}H_2O$ incorporation has been used to measure absolute rates of lipid synthesis in vivo (Baker et al., 1978), the rates of tritiated water incorporation presented in Table 12 should be considered only relative rates for several reasons. First, the rates were calculated from the specific activity of the plasma water, which was fully equilibrated with the tissue (liver) water as indicated by equal dpm/ml in both compartments. However, the total dpm in the body water (approx. 70% of body weight) was only approx. 1% of the total dpm injected into the eel. From preliminary experiments, and the dpm ^{3}H in the tank water, it appears that the $^{3}H_2O$ is freely exchanged between the eel and its environment. Thus, there is a continual decrease in the specific

TABLE 11

The effect of starvation on precursor incorporation into different lipid fractions[1]

Precursor/ Condition	(n)	% FA	% Glycerol	% NS
Glucose+ lactate+ $^{3}H_2O$				
Fed	31	66.3 ± 3.1	29.8 ± 3.1	4.1 ± 0.3
Fasted 4 mo	3	34.5 ± 7.4	63.1 ± 6.8	2.4 ± 0.7
Starved 9 mo	3	24.2 ± 2.1	75.4 ± 2.0	0.4 ± 0.7
Starved 22 mo	1	6.2	93.8	0
Glucose+ 3-^{14}C-lactate				
Fed	11	61.2 ± 9.3	35.1 ± 9.0	2.3 ± 0.2
Starved 9 mo	3	26.5 ± 10.0	71.8 ± 10.0	1.7 ± 0.2
Starved 22 mo	1	18.8	79.6	1.6
Glucose+ lactate+ 1-^{14}C-acetate				
Fed	5	97.1 ± 0.1	0.6 ± 0.1	2.3 ± 0.2
Starved 9 mo	3	90.7 ± 2.6	5.2 ± 2.3	4.1 ± 0.3
Starved 22 mo	1	82.8	15.8	1.4

[1]Precursor label in fatty acids (FA), glycerol, or non-saponifiables (NS) X 100/precursor label in total lipid extract (mean fraction % ± SEM). All mean percentages of FA and glycerol from eels fasted 4 months ($^{3}H_2O$) or starved 9 months (^{14}C) are significantly different ($p<0.05$) from fraction percentages from fed eels.

TABLE 12

The effect of starvation on the in vivo incorporation of ^3H_2O into fatty acids and non-saponifiables in eel liver

	Fed (n=5)	Starved (n=6)
^3H_2O ⟶ Fatty acids[1]	1731.9 ± 748.0	54.8 ± 11.5 **
^3H_2O ⟶ Non-saponifiables	24.7 ± 5.7	10.3 ± 4.4 *
% Wet weight of liver:		
Fatty acids	3.9 ± 0.7	5.8 ± 1.1
Non-saponifiables	0.6 ± 0.1	2.7 ± 1.0 **
Total lipid weight[2]	58.3 ± 12.5	62.3 ± 16.0
HSI[3]	1.29 ± 0.14	0.73 ± 0.01**

Eels were injected i.p. with 0.075 mCi/g of ^3H_2O and killed 30 hr later. The temperature of the tank water was 15°C. Starved animals were not fed for 10 months. [1]µmoles ^3H_2O incorporated into lipid fraction/30 hr/g wet weight of liver (mean±SEM), based on specific activity of plasma water after 30 hr. [2]mg lipid/liver/100 g eel (mg lipid x HSI). [3]Hepato-somatic index, liver weight x 100/body weight. Significance of differences between means, * $p<0.1$; ** $p<0.05$.

activity of the eel body fluids with time, resulting in artificially high rates of tritium incorporation if these are calculated, as in Table 12, from the specific activity at the end of the experiment, since the rate equals dpm FA/specific activity of plasma water. Depending on the rate of $^{3}H_2O$ exchange, the overestimate could be quite large. Secondly, newly formed fatty acids may be utilized for energy (Table 13) or exported from the liver (Table 3). This would result in an underestimate of synthesis in Table 12.

The data in Table 12 confirm two trends that were previously observed with regard to the eels in the experiments reported in Table 5. First, the quantity of lipid is greater in the liver of 9 month starved eels than in fed fish. This is true whether expressed as percentage or as absolute values. Unlike the experiments in Table 5, however, neither is statistically significant ($p>0.05$) due largely to individual animal variability. When the non-saponifiable lipids were measured, compared to fed eels, the starved eels had 5-times more non-saponifiables in terms of percentage, and nearly 3-times more in terms of absolute weight (mg/liver/100 g body weight). Secondly, despite the maintenance of tissue lipids in starved animals, the liver weight (HSI) decreased significantly (Table 12).

IV. UTILIZATION OF LIPIDS: EFFECTS OF STARVATION

Fatty acid synthesis is diminished (Fig. 4, Table 12) in fasted and starved eels, at least in the liver and whole eel side (% weight, Table 5; Table 12). To establish whether these results were due to a decrease in the ability of the liver of starved eels to utilize fatty acids, the production of CO_2 from 1-^{14}C-oleate was estimated in liver slices from fed and starved eels (Table 13). Contrary to expectations, starved eel

TABLE 13

Oxidation of exogenous fatty acid by eel liver slices.

	Fed (n=6)	Starved (n=4)
^{14}C-oleate ——> $^{14}CO_2$[1]	8.9 ± 1.3	22.3 ± 2.1 ***
HSI[2]	1.23 ± 0.09	1.01 ± 0.07 *

Oxidation of 1-^{14}C-oleate (specific activity 56.3 mCi/mmole; 2 µCi/flask) to $^{14}CO_2$ is expressed as picomoles/100 mg wet weight liver/2 hr (mean ± SEM). [2]Hepato-somatic index, liver weight X 100/body weight. Incubation temperature was 15°C. The starved eels were deprived of food for 10 months. Significance of differences between means, * $p<0.1$; *** $p<0.001$. An additional starved eel (not included above) had 10-times the CO_2 production of the mean value for the other starved eels.

liver oxidized oleate more than 2-times faster than fed eel liver, indicating that a decreased ability to oxidize fatty acids does not appear to be the explanation for the maintenance of hepatic lipid content during starvation.

DISCUSSION

I. TISSUE SITES OF LIPID SYNTHESIS

A recurrent problem in studies of lipogenesis is the identification of the primary site(s) of fatty acid synthesis (Favarger, 1965; Baker et al., 1978). In eels, the liver and intestine were found to be the major sites of de novo fatty acid synthesis based on high rates of labeled precursor incorporation both in vivo (Tables 2,3) and in vitro (Table 3). In addition, both tissues contained active complements of "lipogenic" enzymes (Table 4, Fig. 1).

The liver is the primary lipogenic tissue in birds (Pearce, 1980) and man (Shrago et al., 1971), but in most other higher vertebrates studied, adipose tissue is more important (Vernon, 1980). Both liver and adipose tissue contribute to fatty acid synthesis in laboratory rodents (Masoro, 1977; Baker et al., 1978) and frogs (Baranska and Wlodawer, 1969). In fish, however, as confirmed by the present work and others, the liver is invariably the tissue most active in the de novo synthesis of fatty acids based on label incorporation studies (northern pike, *Esox lucius*, Kluytmans and Zandee, 1974; Amazon catfish, Patton et al., 1978; coho salmon, *Oncorhynchus kisutch*, Lin et al., 1977c) or enzyme activities (*Rhodeus amarus*, Braun et al., 1970; white sucker, *Catostomus commersoni*, goldfish, *Carassius auratus*, northern pike, Aster, 1976; coho salmon, Lin et al., 1977a).

Formation of esterified lipids and lipoproteins from fatty acids, monoacylglycerol, cholesterol, and glycerol-1-phosphate in the intestine of fish appears to be similar to that in mammals (Patton et al., 1978). To this author's knowledge, however, the present study is the first

report of the intestine as an important site of de novo fatty acid synthesis in fish. Franks et al. (1966) and Shakir et al. (1978) have reported fatty acid synthesis in rat intestine in vitro, but at lower rates than those presented in Table 3. Favarger (1965), however, in reviewing early label incorporation studies, concluded that the intestine was of equal importance to the liver in those mamallian species for which data was available. In two previous studies of the comparative abilities of different tissues in fish to incorporate labeled acetate into lipids in vivo (northern pike, Kluytmans and Zandee, 1974; Amazon catfish, Patton et al., 1978), both found the liver and gills, and often several other tissues, to contain far greater dpm lipid/mg than the intestine.

Despite the greater in vivo label incorporation in the intestine compared to the liver (Table 2), the in vitro studies would seem to indicate that the liver is the more active of the two tissues in synthesizing fatty acids (Tables 3,4, Fig. 1). In each type of in vitro experiment (acetate or ^3H_2O incorporation, fatty acid synthetase activity (tissue homogenates) or total potential NADPH production), the liver's synthetic ability was always approximately three times that of the intestine.

It is not possible, from the data presented, to be certain which of the two tissues is more important in vivo. One can conclude, however, from the consistent results in the different types of in vitro experiments, that the liver is certainly the more important tissue in vitro, and this agrees with previous in vivo work on other species (northern pike, Kluytmans and Zandee, 1974; Amazon catfish, Patton et al., 1978). Nonetheless, the eel intestine must be considered a significant

source of fatty acids, and one which, due to its anatomical location, is likely to export newly formed lipids to other tissues. Further work on the integration of fish intestinal metabolism with that of other tissues is certainly warranted.

Since visceral fat and red muscle contain a high percentage of lipid (Appendix 5), they were considered to be potential sites of fatty acid synthesis. However, neither tissue incorporated significant amounts of labeled acetate into fatty acids in vivo (Tables 2,3), nor in vitro (red muscle, Table 3; visceral fat, p. 41). The low lipogenic enzyme activities (Table 4), including no detectable ATP-citrate lyase in red muscle, lend further support to the contention that visceral fat and red muscle are not capable of fatty acid synthesis in eels.

Adipose tissue capable of fatty acid synthesis has not been reported in fish, although trout mesenteric adipocytes demonstrate a high fatty acyl-transferase activity presumably for esterification (Henderson and Sargent, unpubl., quoted in Sargent and Henderson, 1980), which suggests that this tissue may represent an energy storage site, as previously suggested by Lin et al. (1977b) for coho salmon. However, visceral fat was found in less than 10% of the yellow eels examined in the present study, and then only in small amounts (< 200 mg). This tissue, therefore, apparently does not store significant energy in eels of this size. In these yellow eels, a primary area of fat storage may be subcutaneous lipid, which is particularly abundant along and between the fibers of the red muscle (Appendix 5). Fish red muscle has been shown to contain active fatty acid oxidizing systems (Bilinski, 1963; Bilinski and Lau, 1969), and it is often found that fish lipids are stored near the sites of utilization (Tashima and Cahill, 1965).

Extra-hepatic tissues, other than the intestine, which appear to be capable of significant rates of de novo lipogenesis are the skin, gills, and brain (Tables 2,3). The skin lipids may contribute, in part, to the subcutaneous lipid stores, although the primary function of skin lipogenesis is probably membrane lipid production, as suggested by Saxena and Zandee (1971), who studied skin lipid synthesis in the cyprinid, *Scardinius erythrophthalmus*.

The ability of the gills to incorporate acetate into lipids, particularly fatty acids, in vitro was somewhat unexpected. Previous workers (Kluytmans and Zandee, 1974) found fatty acid synthesis in northern pike gills to be low, and concluded that the labeled lipids found there were transported from the liver. They also found that a large percentage of the labeled lipids were non-saponifiables, a finding that is confirmed both in vivo and particularly in vitro in this present work. Gills incubated in vitro had the highest fraction percent of labeled non-saponifiables of all the eel tissues studied (about 35%, data not presented). Meister et al. (1976) found active phospholipid synthesis in European eel gills in vivo. One can conclude from previous work that the fish gills contain active synthesizing systems for the assembly of membrane lipids, of which cholesterol and phospholipids are an integral part. What the present work demonstrates is that the gills can produce de novo the "building blocks", non-saponifiables and fatty acids, and do not necessarily rely on transported lipids for these materials; although the percentage of fatty acids and cholesterol formed in situ compared to those transported from other tissues is unknown.

A more detailed look at the class composition of eel lipids from selected tissues, and the relative label incorporation into each class,

based on a limited number of animals, is presented in Appendices 3 and 4. These data are similar to those found in other lean fishes (Kluytmans and Zandee, 1974). However, there is a particularly high amount of label incorporation into the fatty acids of the phospholipid fraction (Appendix 3). This probably reflects the high turnover rate of phospholipids in fishes (Zwingelstein et al., 1975; Meister et al., 1976), or could indicate, as Wilkins (1967) has suggested for herring, *Clupea harengus*, that there is a labile pool of phospholipids which may be used for energy.

In summary, labeled precursor studies, both in vivo and in vitro, supported by enzyme inventories, demonstrated that the liver and intestine are primary sites of de novo fatty acid synthesis, and that other sites, such as skin, gill, and brain and other "visceral tissues" can also produce lipids de novo. Muscle tissue apparently is incapable of this process. However, the interrelationships between the various lipogenic tissues and the lipid storage sites (subcutaneous lipid and the liver; Appendix 5) are unclear and require further study.

II. HEPATIC LIPOGENESIS

A. Lipogenic enzymes, diet, and reducing equivalents.

In the liver, IDH was the most active NADP-linked enzyme (Table 5). Only traces of malic enzyme could be detected and ATP-citrate lyase activity was also low, no more than 15% of the aconitase activity (Table 5). The low activity of ME and ATP-citrate lyase compared to the relatively active cytoplasmic aconitase (but non-detectable mitochondrial aconitase) and IDH, suggests that the citrate cleavage pathway is not operable, and that the predominate fate of citrate

formed in the mitochondria is conversion to isocitrate in the cytoplasm by aconitase. Supporting this conclusion, it was found that aconitase has a 20-fold greater affinity for citrate when compared with ATP-citrate lyase (Table 6). Once isocitrate is produced, NADPH formation proceeds in the cytoplasm or mitochondria depending on the energy state of the cell (Moon and Ouellet, 1979). Thus, any anapleurotic substrate may provide the reducing power for fatty acid synthesis through IDH under lipogenic conditions, and the carbon sources for fatty acid synthesis are probably those that do not require citrate cleavage to produce extramitochondrial acetyl CoA.

Additional NADPH in the liver cytoplasm may be produced by the pentose phosphate pathway, as both G6PDH and 6PGDH are active (Tables 4,5).

Since all of the enzymes involved in the formation of reducing equivalents are located in the cytoplasm, they probably all "compete" for the same pool of NADP, and are acting in the presence of the same pool of the product inhibitor NADPH. The similarity of the Ki(NADPH) values (Table 6) do not enable one to chose between pathways (pentose phosphate vs. citrate cleavage-malate vs. IDH). Furthermore, the significance of the kinetic data may be limited, since, at least in rats, these NADP-linked reactions are near equilibrium (Veech et al., 1969), with the possible exception of G6PDH (Greenbaum et al., 1971). Nonetheless, the apparent affinity of IDH for NADP is the greatest of the four enzymes, and this, coupled with its high specific activity (Table 5) supports an important role for IDH in cytoplasmic NADPH formation in eel liver.

In the intestine (Table 4), the nearly equal activities of the NADP-linked enzymes and the presence of ATP-citrate lyase and aconitase

may reflect the varied substrates this tissue encounters.

The red muscle contained the third highest total NADP-linked enzyme activity (Table 4), with most of this due to IDH. However, there was no detectable ATP-citrate lyase activity, thus precluding the use of glucose, lactate or glucogenic amino acids for lipogenesis in this tissue. The absence of ATP-citrate lyase and the low incorporation of labeled acetate into fatty acids in vivo (Tables 2,3) and in vitro (Table 3) supports the contention that this is not a lipogenic tissue, and the function of its high IDH activity is probably to complete the tricarboxylic acid cycle as proposed for liver NADP-IDH in the eel (Moon and Ouellet, 1979).

The effect of diet on lipogenic enzymes is evaluated for two reasons. First, it was a way to assess the various diets for suitability in further lipogenic studies, and secondly, dietary perturbations, if they reflect changes in carbon flow, can induce changes in key enzymes.

Unlike rats, where the dietary conditions stimulating fatty acid synthesis are reasonably well defined (Romsos and Leveille, 1975), the dietary conditions best suited to the study of fatty acid synthesis in fish are not known, although some work in this area has been done by Lin et al. (1977a,b,c).

The species composition of the eel diet under natural conditions is complex (Sinha and Jones, 1975), but appears to contain some lipid and little carbohydrate. During the colder months (below 10°C) eels undergo a fast and certainly under low temperature laboratory conditions refused to eat (personal observation). In this study, beef liver and worms were used to simulate a natural diet, albeit imperfectly. Annelid

worms are known to constitute a small percentage of the natural diet of some eel populations (Sinha and Jones, 1975).

Reducing equivalent production per liver (Table 5) was highest in the worm-fed eels, and next highest in the freshly caught animals (1977). An important difference between these two groups and the others was the increased activity of G6PDH. However, both the worm-fed and beef-liver fed groups had significantly greater hepato-somatic indices than the corresponding fasted groups. Hence, despite no apparent increase in enzyme activity per gram of liver, the beef-liver fed group showed somewhat greater potential reducing equivalent production. Thus, in either fed group, at least three of the four NADP-linked enzymes did not adapt to changes in dietary conditions, except through increases in total liver weight. Adaptive changes in enzyme activity due to changes in organ size have been reported in rainbow trout, *Salmo gairdneri*, (Buhler and Benville, 1969) and northern pike (Aster, 1976).

The enzymes usually considered to be most responsive to dietary induced changes are G6PDH, ME, and ATP-citrate lyase (Romsos and Leveille, 1974; Lin et al., 1977a,b). However, in these eels G6PDH was the only enzyme to increase in activity per gram in an adaptive manner (Table 5), and only in the worm-fed group. Liver malic enzyme activity was not induced under any condition investigated, and any increased activity of ATP-citrate lyase was accompanied by a concurrent increase in aconitase activity, with the result that no adaptive changes in carbon flow would likely occur. The absence of striking differences in enzyme activities, even in some cases after several months of starvation, is not entirely surprising, since other workers (Buhler and Benville, 1969; Lin et al., 1977a,b) have shown how slowly

fish lipogenic enzymes adapt to dietary changes. Because the eel undergoes a natural winter fast, its enzymes (and subsequent carbon flow) may be less responsive to change than those of other organisms. A similar suggestion has recently been made by French et al. (1981) who found no significant changes in enzyme activities with starvation in trout liver. A diet high in carbohydrates may have resulted in greater lipogenic activity in eels, as in coho salmon (Lin et al., 1977b). It is not likely, however, that a diet high in carbohydrate is relevant to the study of the *usual* processes of fatty acid synthesis in fish (Love, 1970). Dietary considerations aside, the virtual absence of malic enzyme and relatively low activity of ATP-citrate lyase in the eel liver is in direct contrast to the findings of Lin et al. (1977b). They did not, however, measure aconitase activity, so that the precise fate of citrate is not clear.

How common, in fact, is the pattern of high IDH and G6PDH activity and low malic enzyme and ATP-citrate lyase activity in lipogenic tissue (Table 5)? There is, of course, the well known example of ruminants (Ingle et al., 1972). However, domestic cats (Rogers et al., 1977), rabbits (Saggerson, 1974b), and among fish, the carnivorous northern pike (Aster, 1976) appear to follow this pattern. An active malic enzyme in the absence of ATP-citrate lyase in trout liver (Baldwin and Reed, 1976), presents a rather unique situation, but may indicate a mechanism for the transfer of reducing equivalents from the mitochondria to the cytoplasm by malate (Rous, 1978). The common factors in this list appear to be a need to spare glucose (or low glucose availability) and a high input of either amino acids or acetate into the lipogenic tissues. However, the preferential utilization of amino acids as a

carbon source for fatty acid synthesis in carnivorous animals relies, with the exception of leucine (Feller, 1965), on the presence of ATP-citrate lyase. Renaud and Moon (1980a,b) have shown with eel hepatocytes that labeled alanine and aspartate are not incorporated into fatty acids. Since ATP-citrate lyase is required for the incorporation of these amino acids, Renaud and Moon's study indirectly confirms the enzyme data in Table 5. A comparative look at these different patterns of lipogenic enzyme activities is presented in Table 14. This table clearly illustrates how the pattern of enzyme activities in eel liver is quite distinct from that of the rat but closely resembles that of ruminants and rabbits, two animals known to rely on pathways other than citrate cleavage to produce extramitochondrial acetyl CoA (Saggerson, 1974b; Bauman, 1976). The similarity in enzyme complement between ruminants, rabbits, and wholly or partially carnivorous animals (cats and fishes), suggest that the same carbon source, acetate of other ketogenic substrates may be used, and it was to test this possibility that the label incorporation studies were performed.

B. Carbon sources for lipid synthesis.

The direct test of the hypothesis developed from the enzyme results necessitated the demonstration that carbon precursors which require citrate cleavage are incorporated into lipids more slowly than those precursors which form extramitochondrial acetyl CoA without citrate cleavage. For this experiment, a method of assessing fatty acid synthesis independent of the carbon source utilized was required.

The incorporation of ^3H_2O (^3HOH) into lipids has been empirically shown to be a reliable method of estimating the total rate of lipogenesis

TABLE 14

Comparative de novo fatty acid synthesis: Relative synthetic rates of different carbon precursors and relative activities of NADPH-producing enzymes and enzymes involved in citrate metabolism (modified from Saggerson, 1974b; Vernon, 1980).

	Rat Adipose	Guinea Pig Adipose	Rat Liver	Rabbit Adipose	Sheep Adipose	Eel Liver	Cat Liver
Precursor C ⟶ Fatty Acids							
Acetate/Lactate	2.0	1.4	0.3	4.0	3.2	5.3	-
Acetate/Glucose	0.4	3.0	2.5	4.4	30	913	-
Lactate/Glucose	0.2	2.1	8.0	1.1	9.3	172	-
NADPH-producing enzymes:							
NADP-IDH/G6PDH	0.5	0.3	3.3	0.7	2.2	2.5	3.6
NADP-IDH/ME	0.2	2.4	5.0	no ME	40	>100	no ME
Citrate "handling" enzymes:							
NADP-IDH/CCE	0.6	3.3	6.2	10	50	25	60

Data are from the following sources: Rat, guinea pig, rabbit adipose, Saggerson, 1974b; Sheep adipose, Vernon, 1980; Rat liver, Clark et al., 1974, and Taketa et al., 1970; Eel liver, Tables 5 and 9, present work; Cat liver, Rogers et al., 1977. When possible, dietary conditions such as high carbohydrate diets were chosen which emphasized glucose or lactate incorporation.

(Jungas, 1968). Some of the advantages of this method over the incorporation of ^{14}C-labeled substrates are: (1) the rapid penetration and equilibration of $^{3}H_2O$ with intracellular water, (2) the absence of extensive metabolic steps to incorporate the ^{3}H into lipids, and (3) any dilution of the specific activity of labeled water by the generation of unlabeled metabolic water will be negligible (Andersen and Dietschy, 1979). Finally, it has been shown, at least in rat mammary glands, that the rate of $^{3}H_2O$ incorporation is essentially independent of carbon source (Bartley and Abraham, 1976). However, one cannot obtain absolute rates of fatty acid synthesis from tritium incorporation unless one can relate the μg-atoms of ^{14}C incorporated to the number of μg-atoms of ^{3}H.

The relationship between ^{14}C and ^{3}H incorporation into lipids has been studied by Foster and Bloom (1963), Jungas (1968), and reviewed by Rous (1971) and recently by Andersen and Dietschy (1979). For each ^{14}C-acetyl unit of carbon incorporated into newly formed fatty acids, there are potentially four available sites to be labeled by tritium. Several studies (Rous, 1971; Thurmon and Scholz, 1973) have shown that on the even numbered or α-carbons of fatty acids, one hydrogen is derived from H_2O at the enoyl reductase step of fatty acid synthesis, and one is from the carbon substrate. This latter hydrogen is not normally labeled after $^{3}H_2O$ incorporation, since exchange of the substrate carbon-H with water is small. On the odd or β-carbons, hydrogen is from NADPH. Reduced NADP produced by the pentose phosphate pathway cannot exchange with the ^{3}H from tritiated water (Foster and Bloom, 1963; Jungas, 1968), but the other NADPH producing reactions, malic enzyme and NADP-IDH, can produce $NADP^{3}H$ from $^{3}H_2O$ (Rous, 1971). If the pentose phosphate pathway provides all the NADPH required for fatty

acid synthesis, one of the four protons will be labeled, hence 1 $^{3}H/^{14}C_2$, but if this pathway provides none of the reducing equivalents, the ratio is approximately 3 $^{3}H/^{14}C_2$ or 1.5 µg-atom ^{3}H per µg-atom ^{14}C. In most tissues studied to date, the pentose phosphate pathway provides approximately 50-80% of the reducing equivalents, and the ratio $^{3}H/^{14}C$ is 0.87 in rat adipose tissue where the shunt is particularly active and glucose is the main carbon source (Jungas, 1968). In rat liver, where other NADPH producing reactions provide approximately 50% of the reducing equivalents, and carbon sources other than glucose are important (Clark et al., 1974), the ratio of $^{3}H/^{14}C$ is approximately 1.0. Both the pentose phosphate pathway and NADP-IDH provide reducing equivalents for fatty acid synthesis in eel liver (Table 5), although the relative contribution of each is unknown. Therefore, as an estimate, it is assumed that in eel liver one nmole $^{3}H_2O$ incorporated equals one nmole ^{14}C-acetyl unit.

The incorporation of $^{3}H_2O$ into lipids has been followed in two previous fish studies. Changes in lipid synthesis with cold acclimation in rainbow trout hepatocytes were studied by Hazel and Sellner (1979), who found synthetic rates of approx. 5 ng-atoms of tritium incorporated into fatty acids/mg protein/hr in the presence of glucose and lactate at 15°C. Lin et al. (1977c), studying coho salmon, found rates of fatty acid synthesis in liver slices to be approx. 600 nmoles $^{3}H_2O$ incorporated/100 mg liver/hr under comparable conditions. This present study found eel liver slices incorporated approx. 75 nmoles $^{3}H_2O$/100 mg/hr into fatty acids (Fig. 4, fed eels, 2 hr rate divided by 2), which corresponds to approx. 20 ng-atoms/mg protein/hr at 15°C. Lipogenic rates in perfused rat liver (Brunengraber et al. 1973) or rat hepato-

cytes (Clark et al., 1974) range from approx. 4-15 μmoles ^3H_2O incorporated/100 mg wet weight/hr at 37°C.

i. Lactate, glucose, and glucogenic substrates.

When tritiated water was used to measure lipogenesis in eel liver slices, it was found that the addition of lactate plus glucose resulted in the highest rates (Table 7), while the subsequent addition of acetate to this substrate combination did not result in any increase in rates. The addition of glucose with any other substrate resulted in lipogenic rates considerably lower than when lactate was present (Table 7). Thus, although lactate alone was not tested, it is believed that lactate and not glucose is the factor stimulating lipogenesis, although the presence of glucose may be necessary.

The efficacy of the glucose plus lactate combination has previously been demonstrated in trout (Hazel and Sellner, 1979) and rat (Clark et al., 1974) hepatocytes and rat mammary gland slices (Bartley and Abraham, 1976). However, when the nmoles of ^{14}C-glucose and ^{14}C-lactate (as acetyl units) found in fatty acids are totaled and compared with the rates of lipogenesis determined by ^3H_2O incorporation (Table 9), only 10% of the carbon in the newly formed fatty acids is accounted for by the two added substrates in eel liver. Even in the unlikely situation that the mechanisms of tritium incorporation into eel lipids were completely different than those previously found in mammals, the maximum ratio of $^{14}C_2/^3H_2O$ could no more than double accounting for possibly 20% of the carbon. It is clear that despite maximal rates of synthesis when glucose plus lactate are present, the lactate (or glucose) is not providing a significant amount of fatty acid carbon. By contrast, the incorporation of acetyl units compared to tritiated water in rat

mammary gland was near 1:1 for all substrate combinations tested (Bartley and Abraham, 1976), including glucose plus lactate. Here glucose provided approximately 24% and lactate about 71% of the carbon in newly synthesized fatty acids. In rat hepatocytes, in the presence of lactate plus glucose, lactate usually accounted for nearly 80% of the newly incorporated fatty acid carbon, while glucose accounted for 20% or less (Clark et al., 1974).

In eel liver, in vitro, however, lactate is only a minor source of fatty acid (Tables 9,10A,B) or even glycerol carbon (Tables 10A,B), yet lactate is required for maximal rates of lipid synthesis. In rat tissues, where malic enzyme provides reducing equivalents for lipogenesis, NADH derived from lactate by LDH can be transhydrogenated by MDH and ME to form NADPH (Fig. 1A; Pande et al., 1964). However, in the eel, activities of malic enzyme are so low and variable (Table 5) that such a scheme seems unlikely. A possible explanation for this stimulatory effect of lactate addition in eel liver could be NADH production to drive the glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) reaction to produce glycerol-phosphate for esterification. Glycerol-phosphate production is not, however, considered to be rate limiting for lipogenesis (Katz and Wals, 1974).

Despite the finding of Renaud and Moon (1980a) that lactate is an important gluconeogenic substrate, neither their study nor the present one found that lactate (or glucose) contributed significantly to lipid glycerol formation (Tables 10A,B). Somewhat surprisingly, ^{14}C-aspartate formed the highest percentage of lipid glycerol (approx. 70%, data not shown) compared to the total lipid glycerol formed, based on $^{3}H_2O$ incorporation and a ratio of $^{14}C/^{3}H$ of 1:1 (Jungas, 1968). Renaud and Moon

(1980a) have also observed that a considerable portion of utilized aspartate carbon contributes to lipid glycerol formation. It is clear that the flow of carbon into glycerol from glucogenic substrates may proceed through different pathways and it might be expected that the differences encountered may reflect the effect of these different pathways on redox balance (Hanson, 1974; Hayashi and Ooshiro, 1979).

Regardless of the sources of fatty acid or glycerol carbon, the achievement of maximal rates of lipogenesis only when lactate was present (with glucose) emphasizes the importance of lactate or its LDH produced reducing equivalents in the process, and the mechanisms responsible deserve further study.

ii. Endogenous carbon sources.

If the lactate plus glucose combination provides the highest rates of lipid synthesis, but did not provide more than a minor portion of the carbon for fatty acids or glycerol, what was the carbon substrate in these preparations, and why didn't the addition of favored carbon precursors, such as acetate (see below), increase the rate of ^3H_2O incorporation?

Endogenous substrate(s) such as glycogen probably accounts for much of the lipogenesis in the absence of an exogenous precursor in rat tissues (Clark et al., 1974; Salmon et al., 1974). However, glycogen carbon is incorporated into fatty acids through citrate cleavage as is lactate and therefore, it is unlikely to be an important endogenous precursor in eel tissues. The use of endogenous acetate may contribute to the fatty acid carbon, but it is equally likely that a source such as acetoacetate, formed by fatty acid oxidation, may also be important since hepatocytes from the Japanese eel are capable of prodycing this

substrate at rates which are three times greater that the maximum rates of lipogenesis in Table 8 (S. Hayashi, personal communication). Acetoacetate as a lipogenic substrate will be discussed later. It is possible that the endogenous substrates in the presence of glucose plus lactate alone have saturated the lipogenic pathways, and only the presence of citrate, which stimulates acetyl CoA carboxylase (Saggerson, 1980), could increase the rate further (Table 8), although not actually providing additional fatty acid carbon (Table 9). Acetoacetate added to glucose plus lactate did increase the lipogenic rates marginally over glucose plus lactate (Table 8), but the variability of the preparations resulted in an increase that was not statistically significant. If this increase with acetoacetate is real, it is likely to be by mechanisms other than those which occurred with the addition of citrate. The addition of acetate did not stimulate tritium incorporation into lipids, but when acetate was added it was used as a source of fatty acid carbon (Table 9), possibly indicating a shift away from the endogenous source of carbon.

iii. Acetate and acetoacetate.

The significance of the direct "acetogenic" or ketogenic pathways of extramitochondrial acetyl CoA formation compared with those requiring citrate cleavage is best illustrated by Table 9. Labeled acetate and acetoacetate provided the largest percentages of carbon for newly formed fatty acids in the presence of glucose plus lactate in eel liver slices (approx. 70%, Table 9), greatly exceeding the glucogenic precursors. In rat liver, acetate provides a maximum of about 35% of fatty acid carbon, based on $^{14}C/^{3}H_2O$ incorporation in hepatocytes when lactate is not present (Clark et al., 1974), or based on acetate uptake

in perfused liver preparations (Buckley and Williamson, 1977).

The unusual extent to which acetate provides carbon for fatty acid synthesis in eel liver slices is best illustrated in Table 14, which is a comparative summary not only of carbon precursor incorporation, but also of the relative activities of enzymes involved in NADPH production and in the utilization of citrate cleavage based substrates in lipogenic tissues. If one considers rat adipose and ruminant adipose as the two extremes in mammalian lipogenic models, it is then clear, but nonetheless striking, that fatty acid synthesis in the eel liver most closely resembles the ruminant adipose tissue model.

If one proceeds from left to right in Table 14, the higher relative precursor incorporation values indicate a shift from a glucose based to a lactate and then acetate based fatty acid synthesis. In terms of reducing equivalents, the amounts of NADPH produced by the pentose phosphate pathway range from 80-100% in rats and guinea pigs under some conditions (Saggerson, 1974a; Kather and Brand, 1975) to greater use of IDH in rabbits (Saggerson, 1974a) and sheep adipose (Vernon, 1980), and cat liver (Rogers et al., 1977). Rat liver is unusual in essentially having three potential sources of NADPH, but apparently only the pentose phosphate pathway and malic enzymes are utilized (Rous, 1971), and the exact function of NADP-IDH in this tissue is not clear. Lipogenic enzymes of coho salmon liver (not presented in Table 14) show a similarity to the rat liver enzyme pattern (Lin et al., 1977a,b). In the American eel, as previously mentioned (p. 66), the utilization of cytoplasmic IDH is almost obligatory when substrate is oxidized by the tricarboxylic acid cycle under high energy conditions which favor lipogenesis. If sufficient

acetate is activated to acetyl CoA in the mitochondria as well as cytoplasm, then acetate could provide both the carbon source and reducing equivalents (NADPH) necessary for lipid synthesis (Fig. 1B; Ingle et al., 1972).

Even more unusual than its similarity to ruminant adipose tissue in the potential use of acetate as a carbon precursor, and IDH as a source of NADPH, is the ability of eel liver slices to utilize acetoacetate as an important carbon source for fatty acid synthesis in vitro (Table 9). Ketone bodies, particularly acetoacetate, are a quantitatively important source of lipid carbon in the brain of young rats (Edmond, 1974; Yeh et al., 1977; Patel and Clark, 1980) and a probable supplemental source in adult rat and ruminant mammary gland (Williamson et al., 1975). Mouse liver and adipose tissue (Rous, 1976, 1977), and rat adipose tissue (Soling et al., 1970) are capable of utilizing acetoacetate for lipid synthesis. However, in vivo acetoacetate is probably only employed in these tissues as a means to transfer acetyl CoA out of the mitochondrion (Rous and Favarger, 1973; Rous, 1976), since there is a reciprocal relationship between lipogenesis and ketogenesis in these animals (Benito and Williamson, 1978). Liver is generally considered to produce acetoacetate and β-hydroxybutyrate for at least two purposes: as a mechanism for regenerating CoA during periods of extensive fatty acid oxidation, such as starvation, when additional fatty acid oxidation provides energy for gluconeogenesis (Phillips and Hird, 1977b); and, as a mechanism for sparing carbohydrate by providing an alternative substrate in peripheral tissues (Robinson and Williamson, 1980). Eel liver, like liver tissue from most other vertebrates, is capable of producing ketones, primarily acetoacetate

(Phillips and Hird, 1977b; S. Hayashi, personal communication). The utilization of acetoacetate for energy is usually associated with the presence of an active mitochondrial 3-oxoacid CoA transferase (EC 2.8.3.5) which converts acetoacetate to acetoacetyl CoA, which subsequently is converted to acetyl CoA. Most peripheral tissues of mammals (Beis et al., 1980; Robinson and Williamson, 1980) and fish (Beis et al., 1980) (white muscle is an exception) contain an active transferase, but liver tissue of all vertebrates except fishes (Phillips and Hird, 1977b; Zammit et al., 1979) contains low activities of this enzyme.

The high 3-oxoacid CoA transferase activity in the liver mitochondria of eels and other fishes has been explained as a pathway of acetoacetate production rather than utilization, since the presence of this enzyme does not appear to confer upon the eel liver the ability to utilize ketones for energy (Phillips and Hird, 1977b). If so, this pathway is in contrast to the mammalian scheme where acetoacetate is synthesized predominantly through the HMG CoA pathway (Phillips and Hird, 1977b). The transferase may, however, represent a mechanism for the transport of potential CoA derivitives out of the mitochondrion, as postulated by Rous (1976) for mouse adipose tissue and Patel and Clark (1980) for rat brain (Fig. 2). This cytoplasmic acetoacetate could be subsequently activated in the cytoplasm and used for lipogenesis (Buckley and Williamson, 1973). Alternatively, exogenous acetoacetate, from the blood, or added in vitro as in Table 9, could also be activated directly in the cytoplasm by acetoacetyl CoA synthetase as in developing rat brain (Webber and Edmond, 1979) and lactating mammary gland (Buckley and Williamson, 1975). This aceto-

acetyl CoA is either used as a primer for lipogenesis, probably as butyryl CoA (Lin and Kumar, 1972), or converted to acetyl CoA by cytoplasmic acetoacetyl CoA thiolase (EC 2.3.1.9). These possibilities provide mechanisms to explain the incorporation of acetoacetate into lipids by eel liver (Table 9) and may be an alternative explanation for the exceptionally high 3-oxoacid CoA transferase activity in eel liver. Unfortunately, the existance in fish tissues of acetoacetyl CoA synthetase, the enzyme most closely linked with acetoacetate utilization for lipogenesis (Robinson and Williamson, 1980), has not been assessed.

iv. Summary and speculations: Carbon sources.

In summary, the best carbon sources for lipogenesis as determined by labeled precursor incorporation in vitro are those which do not require citrate cleavage to produce extramitochondrial acetyl CoA. These results support the hypothesis based on "lipogenic" enzyme activities. The pattern of precursor incorporation and enzyme activities found for eel liver resembles the adipose tissues of ruminants, which is the acetate-based (ketogenic) extreme of mammalian lipogenic models (Fig. 1B; Table 14), and it is distinctly different from the pattern observed in either rat adipose or rat liver (Table 14). The inability of the eel liver to utilize glucogenic substrates (e.g., lactate, aspartate) for fatty acid synthesis (Table 9) may be a particularly important pattern for carnivorous animals, as well as ruminants, because of their continuous requirements for gluconeogenesis, even in the fed state.

This study is the first, to the author's knowledge, to delineate the pathways of de novo fatty acid synthesis in a lower vertebrate. However, the similarities of these findings to those of lipogenic

tissues of ruminants and rabbits should not necessarily be construed as an indication of a general pattern applicable to all species of fish or lower vertebrates. For example, even though it is well established that ruminant lipogenesis is acetate-based, variations in lipogenic enzyme activities can occur between different breeds of the same ruminant species (Allen et al., 1976), and two recent studies indicate that lactate may be an important carbon source under some conditions (Whitehurst et al., 1978; Prior and Scott, 1980).

The carbon sources utilized by the eels in this study were predicted from the pattern of NADP-dehydrogenase enzyme activities and those enzymes concerned with the citrate branchpoint (ATP-citrate lyase and aconitase). Based on this approach, hepatic lipogenesis in coho salmon may more closely resemble that of rats in vitro, as suggested by Lin et al. (1977b), and utilize primarily glucogenic rather than ketogenic precursors. Furthermore, based on the ratio of hepatic G6PDH activity to IDH activity and the presence or absence of malic enzyme, the data of Aster (1976) may indicate that goldfish and white sucker resemble the lipogenic pattern of coho salmon and rats. In contrast, the non-fatty carnivorous northern pike, with no detectable malic enzyme and ten times greater IDH than G6PDH activities, may synthesize fatty acids utilizing the pathways described for the eels in the present study. Clearly, further comparative work is required in this area.

Although this study demonstrated that acetate and acetoacetate are significant carbon sources for fatty acid synthesis in vitro, can they be considered realistic substrates in vivo? The substrate concentrations (2 to 5 mM) used to directly determine the carbon source

in vitro were considerably higher than those found in vivo (Table 15) and clearly established the *potential* use of these precursors and pathways rather than their actual use.

The acetate concentrations found in eel blood and liver (Table 15) are similar to those of lactate, and clearly no objection can be made to acetate utilization for lipogenesis in vivo based on blood and tissue concentrations of this precursor.

Unfortunately, the assessment of in vivo utilization of a particular precursor is difficult. For example, although the in vivo incorporation of labeled acetate into fatty acids greatly exceeded that of labeled glucose in eels (Table 3), the unknown pool size of unlabeled precursor and number of compartments, plus the competing pathways of metabolism, make interpretation of the in vivo results rather speculative; nonetheless, they are in agreement with the results of the in vitro experiments (Table 9).

If one considers the ability of different mammalian species to incorporate various lipogenic precursors (Table 14), it is seen that the in vivo concentrations of glucogenic and ketogenic precursors presented in Table 15 do not in themselves provide any means of evaluating their significance. For example, in young rats, plasma concentrations of glucose, β-hydroxybutyrate, and acetoacetate decrease in that order, but are utilized for lipid synthesis in the brain in the reverse order, and acetoacetate has the shortest half-life of the three in the blood (Webber and Edmond, 1979). Acetate also has a high turnover rate in monogastric mammals (Knowles et al., 1974). Furthermore, both acetyl CoA synthetase and acetoacetyl CoA synthetase from mammals have apparent Km values which are similar to the in vivo

TABLE 15

Concentrations of potential lipogenic precursors in the blood and/or liver of eels, rats, and ruminants.

	Precursors			
	Acetate	Acetoacetate	Lactate	Glucose
Eel:				
Blood/Plasma	0.33[1]	0.04[2]	0.30[3]	3.5[1]
Liver	0.32[1]	-	0.32[3]	6.4[3]
Rat:				
Blood/Plasma	0.20[4]	0.03[2]	1.23[5]	6.3[6]
Liver	0.63[4]	0.06[7]	0.84[7]	5.4[7]
Ruminant:				
Blood/Plasma	0.63[4]	0.04[8]	0.55[8]	2.7[8]
Liver	-	-	-	-

All values are for fed animals except for the eel acetoacetate value, which is from 3-4 wk fasted fish. Acetoacetate comprises approx. 30-40% of the ketones in rat blood (Phillips and Hird, 1977b), but only 10% of the ketones in ruminant blood (Baird, 1977). Concentrations are expressed as mM in whole blood or plasma, and as μmoles/g wet weight in liver. Data is from the following sources (superscripts in Table): [1]Estimated in this laboratory as described in Materials and Methods, *A. rostrata*; [2]Phillips and Hird, 1977b, *A. australis;* [3]Renaud and Moon, 1980b, *A. rostrata;* [4]Knowles et al., 1974, rat, sheep; [5]Phillips and Hird, 1977a, *A. australis;* [6]Hawkins et al., 1971, rat; [7]Greenbaum et al., 1971, rat; [8]Baird, 1977, dairy cow.

concentrations of their respective substrates, which may, in part, explain the efficient utilization of these precursors despite their low concentrations.

If one assumes that the high relative turnover rates of mammalian blood acetate and acetoacetate are representative of the situation occurring in eels, the source of these precursors must be considered. Phillipson (1947) and Buckley and Williamson (1977) have shown that acetate can be produced in large quantities by the intestinal gut flora in fed, monogastric animals. Sera and Ishida (1972) and Ugajin (1979) have shown that the gut flora of fishes also produces acetate. The acetate produced in the intestine is transported to the liver, and Buckley and Williamson (1977) concluded that the primary fate of this exogenous precursor is lipid synthesis. The intestine may likewise be a major source of acetate for hepatic lipogenesis in the eel and this certainly requires further investigation.

Blood acetoacetate levels (Table 15) may bear no relationship to the utilization of this precursor for hepatic lipogenesis since the liver is the primary source of blood acetoacetate (Robinson and Williamson, 1980). If acetoacetate production is great enough in the fed state to maintain a rapidly metabolized blood concentration of 0.04 mM in the eel (Table 15), then it may be high enough to supply acetoacetate for hepatic lipogenesis.

To fully assess the validity of the ketogenic vs. glucogenic precursor models (Fig. 1A,B) one must also consider the potential changes at different stages of the animal's life history. It was found (data not presented) that the incorporation of ^{14}C-glucose into fatty acid carbon was much greater in eels weighing less than

60 g (mean, 45 g) than in animals weighing 90 g or more (the latter data presented in Table 3). If weight or some weight dependent character makes a considerable difference in the ability to utilize certain precursors, it is apparent that an evaluation of carbon sources or rates of lipogenesis should be made at several stages of the animal's life history. Furthermore, both the glucose incorporation data and the presence of low amounts of ATP-citrate lyase and malic enzyme in liver indicate that the eel has the genetic potential to utilize glucogenic carbon precursors for hepatic lipogenesis, but at least in vitro, the eel appears "to chose not to do so." Clearly then, the use of the ketogenic pathways for generation of fatty acid carbon must confer an advantage to the animal, and, as others have also suggested (Jones and Wahle, 1980), this probably reflects the input or availability of substrates to the lipogenic tissues and the competition for carbon that may arise between the different pathways, such as gluconeogenesis and lipogenesis. From this present study, eels seem to have solved the problem of competition for carbon, but further work is required to show that in vivo the "choice" of ketogenic substrates is a realistic one based on substrate supply.

The evaluation of the in vivo significance of the different precursors has been rather speculative, but these speculations postulate no new mechanisms, and are clearly within the framework of accepted metabolic limitations in other species (see Appendix 6, p. 111).

C. Lipogenesis, energy source, and relationship to gluconeogenesis and ketogenesis.

Although there are similarities between eel liver and ruminant adipose tissue with regard to fatty acid synthesis (Table 14), the

latter organ is designed strictly as an energy storage tissue (Ballard et al., 1969), and not as a "metabolic integrator" with the multiplicity of functions characteristic of hepatic tissue. Gluconeogenesis and ketogenesis are important additional functions of liver tissue, and each of these is a potential competitor with lipogenesis for carbon, reducing equivalents, and energy. In fed eels, lipogenesis (this study) and gluconeogenesis (Renaud and Moon, 1980a,b) occur simultaneously, since glucose needs to be supplied continually due to its near absence in the diet (Table 1). This is contrary to the situation in some rodents where a reciprocal relationship exists between the two pathways (Tepperman and Tepperman, 1970). The absence of competition between thses two pathways for carbon in eel liver has previously been discussed (see above).

Regarding reducing equivalents, there is no direct competition since NADPH is required for fatty acid synthesis while gluconeogenesis is acutely affected by the NAD redox couple. Unfortunately, it is not known how gluconeogenesis in eels effects or is affected by the redox state during periods of increased gluconeogenesis during starvation. Furthermore, β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), an enzyme involved in adjustments of redox state during active fatty acid oxidation and fasting (Robinson and Williamson, 1980), is absent from fish liver (Zammit et al., 1979). This observation implies that gluconeogenesis may be (1) insensitive to increased NADH, (2) that increases in fatty acid oxidation, a source of mitochondrial NADH, are not sufficient to effect redox potential and/or (3) that eel liver oxidizes NADH through other reactions. Further studies are required to assess these possibilities.

Since both gluconeogenesis and lipogenesis are anabolic processes, do they compete for energy in the form of ATP? Renaud and Moon (1980b) have shown that mitochondrial glucogenic substrate oxidation is positively correlated with, and is just sufficient to fuel glucose production, rather than fueling this process by fatty acid oxidation, as other workers have suggested (Phillips and Hird, 1977a; S. Hayashi, personal communication). When fatty acid synthesis is based on exogenous glucose, or a combination of glucose and lactate, as in rat adipose tissue, the lipogenic process itself supplies enough energy (Flatt, 1970). In fact, Katz and Wals (1974) found that there was a sizeable excess ATP production under in vitro lipogenic conditions. In ruminant adipose tissue and mammary gland, acetate can be utilized as a source of both lipid carbon and energy (Ingle et al., 1972), and some simultaneous oxidation of lactate or glucose may be assumed. However, liver tissue of most vertebrates has a R.Q. (respiratory quotient) of near 0.7 (Phillips and Hird, 1977b), indicating that fatty acids are the primary substrate oxidized. Hence in the fed state in the liver of eels and other vertebrates with hepatic lipogenic capabilities, fatty acid oxidation and synthesis may be proceeding simultaneously. Indeed, in the liver of fed rats, ketogenesis, indicative of fatty acid oxidation, proceeds at 17% of the rate of lipogenesis (Brunengraber et al., 1973). It is postulated that a low level of fatty acid oxidation in the fed liver may provide much of the energy for de novo fatty acid synthesis, since production of ATP as a result of the complete oxidation of fatty acids far exceeds that required for equimolar synthesis, given a source of exogenous carbon. Hazel and Sellner (1979) have suggested that fatty acid synthesis may

supply substrates to support fatty acid oxidation, which would, in turn support further lipogenesis in fed, cold-acclimated trout liver where lipid stores are low but rates of lipogenesis high.

Furthermore, it is suggested that in the eel liver, the incomplete oxidation of a portion of the fatty acids may produce some of the substrate used for fatty acid synthesis, especially acetoacetate. This material would be transported from the mitochondria to the cytoplasm to supplement other potential exogenous lipogenic substrates such as acetate, or to a minor extent, lactate. The use of acetoacetate as a lipogenic substrate, or as a general mechanism in the transport of carbon from the mitochondrion to the cytoplasm in eel liver was discussed above (p. 79) and may explain the function of the high activity of acetoacetyl CoA thiolase and 3-oxoacid CoA transferase found by Phillips and Hird (1977b) and Zammit and Newsholme (1979) in fish. The use of fatty acids oxidized to the two or four-carbon level for energy or reutilized for lipid carbon must require a tightly controlled balance between fatty acyl CoA and malonyl CoA concentrations, since they reciprocally inhibit the aforementioned anabolic and catabolic processes, respectively (McGarry and Foster, 1980).

III. STARVATION: SYNTHESIS AND UTILIZATION OF FATTY ACIDS

Food-deprivation for four months or more caused a marked decrease in fatty acid synthesis in eels regardless of whether the labeled precursor was $^{3}H_2O$, ^{14}C-acetate, or ^{14}C-lactate (Fig. 4, Tables 10A,B, 12). This significant drop in fatty acid synthesis was in contrast to the relatively small change in "lipogenic" enzyme activities (Table 5) and the maintenance of lipid reserves (Tables 5, 12) during fasting (4 months)

or longer term starvation (>4 months). Furthermore, despite the decrease in fatty acid synthesis, precursor incorporation into lipid glycerol actually increased with a four month fast, followed by a slow decrease with more extended periods of food-deprivation (Fig. 4). This latter finding confirms a similar increase in precursor incorporation into lipid glycerol during a five month fast in American eel hepatocytes reported by Renaud and Moon (1980b). It is clear from the present work, however, that label incorporation into the glycerol fraction of saponified lipid extracts is by no means an indication of increased rates of lipid synthesis. The decreased rate of fatty acid synthesis during fasting is consistant with the findings in mammals (Masoro, 1977) and coho salmon (Lin et al., 1977c) but unlike the northern pike (Kluytmans and Zandee, 1974) where no decrease in ^{14}C-acetate incorporation into lipids occurred after two months of starvation.

Although lipid synthesis decreased during starvation, the percentage of total lipid in muscle, and total lipid, fatty acids, and cholesterol in liver increased during starvation (Tables 5,12). Particularly marked was the five-fold increase in liver cholesterol (non-saponifiables, Table 12). The increase in cholesterol and total lipid with starvation was absolute (mg/liver, Table 12). A much smaller increase in percent liver cholesterol during starvation has been reported in *A. anguilla* (Dave et al., 1975). The increased lipid percentages in liver and muscle suggest that other substances such as protein or carbohydrates may be utilized preferentially for energy during periods of food-deprivation. During extended periods of starvation (>1 year) the eels in this laboratory develop an emaciated appearance, and it is

apparent that the muscle mass is being depleted. The carbohydrate content of muscle is low (Renaud and Moon, 1980b), and lipid percentage increases (Table 5), so it is primarily muscle protein and/or whole tissue (Wilkins, 1967) that is used for energy during starvation in eels of this size. This is also the conclusion reached by Lovern (1939), Butler (1968), and Renaud and Moon (1980b).

The use of peripheral protein for energy and to maintain carbohydrate reserves is not surprising, since these eels are relatively lean (non-fatty) fish at this stage in their life history and do not have large lipid stores compared to more mature animals (personal observations). Muscle protein constitutes the largest energy store in the body, since the muscle mass is at least 80% of the body weight. Furthermore, the swimming activity of eels decreased with starvation in the laboratory, as it does during the over-wintering fast (Nyman, 1972). Hence there is less need for muscle tissue, and its utilization may not be detrimental. According to Love (1970), the sources of energy utilized and depleted during starvation reflect, in part, the amount and tissue sites of lipid storage. In older yellow eels, and in mature, migrating silver or bronze eels, the lipid reserves associated with the visceral cavity and subcutaneous regions are larger (Lewander et al., 1974; personal observation). The mature eel is thus more likely to utilize these reserves, just as northern pike have been shown to utilize visceral fat first during starvation (Ince and Thorpe, 1976) when this reserve is present and whole muscle when it is not (Medford, 1976).

Other eel studies have found that protein is not utilized to the extent suggested by the present work. Larsson and Lewander (1973) found that eels (*A. anguilla*) depleted liver triacylglycerols quickly while muscle triacylglycerols were used concurrently with muscle

protein after prolonged (6 months) starvation, and there was an increase in plasma free fatty acids (FFA). Dave et al. (1975), however, using the same species, found that liver and muscle triacylglycerols decreased from 47 to 96 days of starvation, but plasma FFA, though variable, did not increase even after 164 days of starvation. Another eel species, *A. japanica* (Inui and Oshima, 1966), utilizes muscle and liver glycogen, and liver triacylglycerols, rather than protein during moderate starvation.

The reasons for these differences in energy utilization during starvation in different groups of eels are not known. Love (1970) has suggested that it may reflect the amount of lipid available, particularly in the muscle, since lean fish, such as the yellow eels studied in this laboratory, have been shown to spare carbohydrate by utilizing muscle proteins for gluconeogenesis (Butler, 1968; Renaud and Moon, 1980b). Fish with greater lipid reserves in the muscle and visceral cavity tend to utilize this substrate rather than muscle protein (Love, 1970). The eels examined in the present study, and those by Inui and Oshima (1966) and Dave et al. (1975) were all approximately the same size, yet utilized primarily protein, glycogen, or lipid, respectively, during starvation. It is evident that the extent of the energy reserves, and not animal size or close taxonomic position, may be a significant factor in substrate utilization (for further examples, see Love, 1970; Inee and Thorpe, 1976).

During starvation, the synthesis of fatty acids decreased (Fig. 4) but lipid was not depleted (Table 12). One might predict, therefore, that fatty acid oxidation would be significantly decreased to explain these observations. However, hepatic oxidation of oleate increased

significantly during starvation (Table 13). Oleate is a fatty acid associated with active mobilization to meet energy demands (Dave et al., 1976) and comprises a significant proportion of eel liver fatty acid (personal observation). For these reasons it is probably a good indicator of the overall rate of β-oxidation. If so, then there is, at present, no obvious explanation for the maintenance of lipid reserves in the liver during starvation.

Since liver tissue of all vertebrates appears to utilize lipid for energy (Phillips and Hird, 1977b), the increase in fatty acid oxidation with starvation (Table 13) may reflect increased energy demands on the liver, including increased gluconeogenesis which occurs during starvation in eel liver (Renaud and Moon, 1980b). However, the relationship between gluconeogenesis and fatty acid oxidation has not been established in the American eel. Fatty acids stimulate gluconeogenesis in rats (Stucki, 1972) and their oxidation probably provides the energy for glucose production in the Australian (Phillips and Hird, 1977a.b) and Japanese eels (S. Hayashi, personal communication). On the other hand, gluconeogenesis is inhibited by fatty acids in perfused livers of guinea pigs, rabbits, and cats (Hanson, 1974), and it has been postulated by Renaud and Moon (1980b) that concomittant glucogenic substrate oxidation provides much of the energy required for gluconeogenesis.

In summary, during starvation these eels decreased lipid synthesis but maintained lipid reserves and increased fatty acid oxidation in vitro. Clearly, control over fat utilization needs to be investigated further.

IV. SUMMARY

1. In the American eel, *A. rostrata*, the liver and intestine are the tissues most actively involved in fatty acid synthesis. Neither red nor white muscle play an important role in this process.

2. "Lipogenic" enzymes are most active in the liver, and with the exception of G6PDH, are relatively unresponsive to food-deprivation of up to 6 months.

3. In the liver, the high activities of NADP-IDH and aconitase compared to the low activities of citrate cleavage enzyme and malic enzyme suggest that carbon flow from citrate is towards isocitrate and that IDH is an important source of reducing equivalents for fatty acid synthesis.

4. The incorporation of acetate and acetoacetate into fatty acids was far greater than that of glucogenic precursors, both in absolute terms and in comparison with the incorporation of ^3H_2O. Thus, the carbon sources for hepatic fatty acid synthesis in vitro are those which do not require citrate cleavage to produce extramitochondrial acetyl CoA.

5. The overall pattern of de novo fatty acid synthesis in eel liver is similar to that of ruminant adipose tissue and may be an adaptation to spare glucose and glucogenic precursors.

6. Food-deprivation caused a significant decrease in the in vivo and in vitro rates of hepatic fatty acid synthesis. The ability of the liver to oxidize oleate increased with starvation. However, eels maintained their lipid reserves in both liver and muscle during this period. Thus, it is concluded, that muscle protein or whole tissue are utilized for energy under these conditions.

LITERATURE CITED

Allen, C.E., Beitz, D.C., Cramer, D.A. and Kauffman, R.G. (1976) Biology of fat in meat animals. North Central Reg. Res. Publ. No. 234. College of Agriculture and Life Sciences, University of Wisconsin-Madison.

Amenta, J.S. (1964) A rapid chemical method for quantification of lipids seperated by thin-layer chromatography. J. Lipid Res. 5:270-272.

Andersen, J.M. and Dietschy, J.M. (1979) Absolute rates of cholesterol synthesis in extrahepatic tissues measured with ^{3}H-labeled water and ^{14}C-labeled substrates. J. Lipid Res. 20:740-752.

Aster, P.L. (1976) The influence of temperature and seasonal acclimatization on G6PD and 6PGD from three teleost species. M.Sc. Thesis. Univ. of Alberta. 124 pp.

Baird, G.D. (1977) Aspects of ruminant intermediary metabolism in relation to ketosis. Biochem. Soc. Trans. 5:S19-S27.

Baker, N., Learn, D.B. and Bruckdorfer, K.R. (1978) Re-evaluation of lipogenesis from dietary glucose carbon in liver and carcass of mice. J. Lipid Res. 19:S79-S93.

Baldwin, J. and Reed, K.C. (1976) Cytoplasmic sources of NADPH for fat synthesis in rainbow trout liver, effect of thermal acclimation on enzyme activities. Comp. Biochem. Physiol. 54B:527-529.

Ballard, F.J., Hanson, R.W. and Kronfeld, D.S. (1969) Gluconeogenesis and lipogenesis in tissue from ruminant and nonruminant animals. Federation Proc. 28:218-231.

Baranska, J. and Wlodawer. (1969) Influence of temperature on the composition of fatty acids and on lipogenesis in frog tissues. Comp. Biochem. Physiol. 28:553-570.

Bartley, J.C. and Abraham, S. (1976) The absolute rate of fatty acid synthesis by mammary gland slices from lactating rats. J. Lipid Res. 17:467-477.

Bauman, D.E. (1976) Intermediary metabolism of adipose tissue. Federation Proc. 35:2308-2313.

Beis, A., Zammit, V.A. and Newsholme, E.A. (1980) Activities of 3-hydroxybutyrate dehydrogenase, 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase in relation to ketone-body utilisation in muscle from vertebrates and invertebrates. Eur. J. Biochem. 104:209-215.

Benito, M. and Williamson, D.H. (1978) Evidence for a reciprocal relationship between lipogenesis and ketogenesis in hepatocytes from fed virgin and lactating rats. Biochem. J. 176:331-334.

Bilinski, E. (1963) Utilization of lipids by fish I. Fatty acid oxidation by tissue slices from dark and white muscle of rainbow trout. Can. J. Biochem. Physiol. 41:107-112.

Bilinski, E. (1974) Biochemical aspects of fish swimming. In Biochemical and Biophysical Perspectives in Marine Biology (Edited by Malins, D.C. and Sargent, J.R.), pp. 239-288. Academic Press, New York.

Bilinski, E. and Gardner, L.J. (1968) Effect of starvation on free fatty acid level in blood plasma and muscular tissues of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Bd. Canada 25:1555-1560.

Bilinski, E. and Lau, Y.C. (1969) Lipolytic activities toward long-chain triglycerides in lateral line muscle of rainbow trout. J. Fish. Res. Bd. Canada 26:1857-1866.

Braun, K., Kunnemann, H. and Laudien, H. (1970) Der Einfluss von Temperaturanderungen auf Enzyme der Fischmuskulatur. Versuche mit *Rhodeus amarus*. Mar. Biol. 7:59-70.

Brumengraber, E., Boutry, M. and Lowenstein, J.M. (1973) Fatty acid and 3-hydroxysterol synthesis in the perfused rat liver. J. Biol. Chem. 248:2653-2669.

Buckley, B.M. and Williamson, D.H. (1973) Acetoacetate and brain lipogenesis: developmental pattern of acetoacetyl-coenzyme A synthetase in the soluble fraction of rat brain. Biochem. J. 132:653-656.

Buckley, B.M. and Williamson, D.H. (1975) Acetoacetyl CoA synthetase; a lipogenic enzyme in rat tissues. FEBS Lett. 60:7-10.

Buckley, B.M. and Williamson, D.H. (1977) Origins of blood acetate in the rat. Biochem. J. 166:539-545.

Buhler, D.R. and Benville, P. (1969) Effect of feeding and of DDT on the activity of hepatic glucose-6-phosphate dehydrogenase in two salmonids. J. Fish. Res. Bd. Canada 26:3209-3216.

Butler, D.G. (1968) Hormonal control of gluconeogenesis in the North American eel (*Anguilla rostrata*). Gen. Comp. Endocrin. 10:85-91.

Clark, D.G., Rognstad, R. and Katz, J. (1974) Lipogenesis in rat hepatocytes. J. Biol. Chem. 249:2028-2036.

Clarke, S.D., Romsos, D.R. and Leveille, G.A. (1977) Influence of dietary fatty acids on liver and adipose tissue lipogenesis and on liver metabolites in meal-fed rats. J. Nutr. 107:1277-1287.

Creach, Y. and Murat, J.C. (1974) Le jeune et la realimentation chez la carpe (*Cyprinus carpio*)-7. Metabolism du glucose-1-^{14}C et du glucose-6-^{14}C. Arch. Sci. Physiol. 28:157-172.

D'Adamo, A.F., Jr., Gidez, L.I. and Katsu, F.M. (1968) Exp. Brain Res. 5:267-273. (Cited in Patel, T.B. and Clark, J.B. (1980) Biochem. J. 188:163-168.).

Dave, G., Johansson, M.L., Larsson, A., Lewander, K. and Lidman, U. (1974) Metabolic and hematological studies on the yellow and silver phases of the European eel, *Anguilla anguilla* L.-II. Fatty acid composition. Comp. Biochem. Physiol. 47B:583-591.

Dave, G., Johansson, M.L., Larsson, A., Lewander, K. and Lidman, U. (1975) Metabolic and hematological effects of starvation in the European eel, *Anguilla anguilla* L.-I. Carbohydrate, lipid, protein, and inorganic ion metabolism. Comp. Biochem. Physiol. 52A:423-430.

Dawson, R.M.C., Elliott, D.C., Elliott, W.E. and Jones, K.M. (1969) Data for Biochemical Research. Oxford Univ. Press, Toronto.

Dixon, M. and Webb, E.C. (1964) Enzymes. Second Edition. Academic Press, New York.

Edmond, J. (1974) Ketone bodies as precursors of sterols and fatty acids in the developing rat. J. Biol. Chem. 249:72-80.

Favarger, P. (1965) Relative importance of different tissues in the synthesis of fatty acids. *In* Handbook of Physiology, Section 5, Adipose Tissue (Edited by Renold, A.E. and Cahill, G.F., Jr.), pp. 19-23. Amer. Physiol. Soc., Washington, D.C.

Feller, D.D. (1965) Conversion of amino acids to fatty acids. *In* Handbook of Physiology, Section 5, Adipose Tissue (Edited by Renold, A.E. and Cahill, G.F., Jr.), pp. 363-373. Amer. Physiol. Soc., Washington, D.C.

Flatt, J.P. (1970) Conversion of carbohydrate to fat in adipose tissue: an energy-yielding and, therefore, self-limiting process. J. Lipid Res. 11:131-143.

Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Foster, D.W. and Bloom, B. (1963) The synthesis of fatty acids by rat liver slices in tritiated water. J. Biol. Chem. 238:888-892.

Franks, J.J., Riley, E.M. and Isselbacher, K.J. (1966) Synthesis of fatty acids by rat intestine. Proc. Soc. Exp. Biol. Med. 121: 322-327.

French, C.J., Mommsen, T.P. and Hochachka, P.W. (1981) Amino acid utilization in isolated hepatocytes from rainbow trout. Eur. J. Biochem. In Press.

Friedberg, S.J., Harlan, W.R., Trout, D.L. and Estes, E.H. (1960) The effect of exercise on the concentration and turnover of plasma nonesterified fatty acids. J. Clin. Invest. 39:215-220.

Fritz, I.B. and Yue, K.T.N. (1964) Effects of carnitine on acetyl CoA oxidation by heart muscle mitochondria. Amer. J. Physiol. 206: 531-535.

Good, C.A., Kramer, H. and Somogyi, M. (1933) The determination of glycogen. J. Biol. Chem. 100:485-491.

Greenbaum, A.L., Guma, K.A. and McLean, P. (1971) The distribution of hepatic metabolites and the control of the pathways of carbohydrate metabolism in animals of different dietary and hormonal status. Arch. Biochem. Biophys. 143:617-663.

Haddock, B.A., Yates, D.W. and Garland, P.B. (1970) The localization of some coenzyme A-dependent enzymes in rat liver mitochondria. Biochem. J. 119:565-575.

Hanson, R.A. (1974) The choice of animal species for studies of metabolic regulation. Nutr. Rev. 32:1-8.

Hanson, R.W. and Ballard, F.J. (1967) The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissues from ruminants. Biochem. J. 105:529-536.

Hazel, J.R. (1979) The influence of temperature adaptation on the composition of the neutral lipid fraction of rainbow trout (*Salmo gairdneri*) liver. J. Exp. Zool. 207:33-42.

Hazel, J.R. and Prosser, C.L. (1974) Molecular mechanisms of temperature compensation in poikilotherms. Physiol. Rev. 54:620-677.

Hazel, J.R. and Sellner, P.A. (1979) Fatty acid and sterol synthesis in hepatocytes of thermally acclimated rainbow trout (*Salmo gairdneri*). J. Exp. Zool. 208:105-114.

Hawkins, R.A., Williamson, D.H. and Krebs, H.A. (1971) Ketone body utilization by adult and suckling rat brain in vivo. Biochem J. 122:13-18.

Hayashi, S. and Ooshiro, Z. (1979) Gluconeogenesis in isolated liver cells of the eel, *Anguilla japanica*. J. Comp. Physiol. 132:343-350.

Hochachka, P.W. and Somero, G.N. (1973) Strategies of Biochemical Adaptation. W.B. Saunders Co., Philadelphia 358 pp.

Ince, B.W. and Thorpe, A. (1976) The effects of starvation and force-feeding on the metabolism of the northern pike, *Esox lucius* L. J. Fish Biol. 8:79-88.

Ingle, D.L., Bauman, D.E. and Garrigus, U.S. (1972) Lipogenesis in the ruminant: in vitro study of tissue sites, carbon source and reducing equivalent generation for fatty acid synthesis. J. Nutr. 102:609-616.

Inui, Y. and Oshima, Y. (1966) Effect of starvation on metabolism and chemical composition of eels. Bull. Jap. Soc. Scient. Fish. 32:492-501.

Jackson, R.L., Morrisett, J.D. and Gotto, A.M. (1976) Lipoprotein structure and metabolism. Physiol. Rev. 56:259-316.

Jones, C.T. and Wahle, K.W.J. (1980) Aspects of lipid metabolism in the foetal, neonatal and adult monogastric mammal. Biochem. Soc. Transactions. 8:289-290.

Jungas, R.L. (1968) Fatty acid synthesis in adipose tissue incubated in tritiated water. Biochemistry 7:3708-3717.

Kates, M. (1972) Techniques in lipidology. *In* Laboratory Techniques in Biochemistry and Molecular Biology. (Edited by T.S. Work and E. Work) pp 267-601. American Elsevier, New York.

Kather, H. and Brank, K. (1975) Origin of hydrogen required for fatty acid synthesis in isolated rat adipocytes. Arch. Biochem. Biophys. 170:417-426.

Katz, J. and Wasl, P.A. (1974) Lipogenesis from lactate in rat adipose tissue. Biochemica et Biophysica Acta. 348:344-356.

Katz, J., Wals, P.A. and Ven de Velde, R.L. (1974) Lipogenesis by acini from mammary gland of lactating rats. J. Biol. Chem. 249:7348-7357.

Kluytmans, J.H.F.M. and Zandee, D.I. (1974) Lipid metabolism in the northern pike (*Esox lucius* L.)-3. In vivo incorporation of 1-^{14}C-acetate in the lipids. Comp. Biochem. Physiol. 48B:641-649.

Knipprath, W.G. and Mead, J.F. (1968) The effect of the environmental temperature on the fatty acid composition and on the in vivo incorporation of 1-^{14}C-acetate in goldfish (*Carassius auratus* L.). Lipids 3:121-128.

Knowles, S.E., Jarret, I.G., Filsell, O.H. and Ballard, F.J. (1974) Production and utilization of acetate in mammals. Biochem J. 142:401-411.

Kornacker, M.S. and Lowenstein, J.M. (1965) Citrate and the conversion of carbohydrates into fat. Biochem. J. 94:209-215.

Larsson, A. and Lewander, K. (1973) Metabolic effects of starvation in the eel, *Anguilla anguilla* L. Comp. Biochem. Physiol. 44A:367-374.

Leslie, J.M. and Buckley, J.T. (1976) Phospholipid composition of goldfish (*Carassius auratus* L.) liver and brain and temperature dependence of phosphatidyl chlorine synthesis. Comp. Biochem. Physiol. 53B:335-337.

Lewander, K., Dave, G., Larsson, A. and Lidman, U. (1974) Metabolic and hematological studies on the yellow and silver phases of the European eel, *Anguilla anguilla* L. I. Carbohydrate, lipid, protein, and inorganic ion metabolism. Comp. Biochem. Physiol. 47B:571-582.

Lin, C.Y. and Kumar, S. (1972) Pathway for the synthesis of fatty acids in mammalian tissues. J. Biol. Chem. 247:604-610.

Lin, H., Romsos, D.R., Tack, P.I. and Leveille, G.A. (1977a) Influence of dietary lipid on lipogenic enzyme activities in coho salmon, *Oncorhynchus kisutch* (Walbaum). J. Nutr. 107:846-854.

Lin, H., Romsos, D.R., Tack, P.I. and Leveille, G.A. (1977b) Effects of fasting and feeding various diets on hepatic lipogenic enzyme activities in coho salmon (*O. kisutch* (Walbaum)). J. Nutr. 107:1477-1483.

Lin, H., Romsos, D.R., Tack, P.I. and Leveille, G.A. (1977c) Influence of diet on in vitro and in vivo rates of fatty acid synthesis in coho salmon (*O. kisutch* (Walbaum)). J. Nutr. 107:1677-1682.

Love, R.M. (1970) The Chemical Biology of Fishes, pp. 202-203, Academic Press, New York.

Lovern, J.A. (1939) Captive eels. Some observations on their behaviour. Salm. Trout Mag. 94:56-57.

Lowry, O.H., Rosebraugh, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Maddy, A.H. and Spooner, R.O. (1970) Ox erythrocyte agglutinability. 1. Variation in the membrane protein. Vox Sang. 18:34-41.

Masoro, E.J. (1977) Lipids and lipid metabolsim. Ann. Rev. Physiol. 39:301-321.

McGarry, J.D. and Foster, D.W. (1980) Regulation of hepatic fatty acid oxidation and ketone body production. Ann. Rev. Biochem. 49:395-420.

Medford, B.A. (1976) Annual cycle in condition and nutrient composition of northern pike (*Esox lucius* L.). M.Sc. Thesis, Univ. of Alberta, Edmonton, Alberta 61 pp.

Meister, R., Zwingelstein, G. and Brichon, E. (1976) Influence de la température d'acclimation sur le métabolisme des phospholipides de l'anguille (*Anguilla anguilla*) en eau douce. J. Physiol. Paris. 72:79-103.

Moon, T.W. and Ouellet, G. (1979) The oxidation of tricarboxylic acid cycle intermediates, with particular reference to isocitrate, by intact mitochondria isolated from the liver of the American eel, *Anguilla rostrata* LeSueur. Arch. Biochem. Biophys. 195:438-452.

Mulder, I. and van den Bergh, S.G. (1977) Conversion of acetoacetyl-coenzyme A into acetoacetate in subcellular liver preparations. Int. J. Biochem. 8:227-235.

Narayansingh, T. and Eales, J.G. (1975) The influence of physiological doses of thyroxine on the lipid reserves of starved and fed brook trout, *Salvelinus fontinalis* (Mitchell). Comp. Biochem. Physiol. 52B:407-412.

Nyman, L. (1972) Some effects of temperature on eel (*Anguilla*) behavior. Inst. Freshwater Res. (Drottningholm, Sweden) 52:90-102.

Pande, S.V., Kahn, R.P. and Venkitasubramanian, T.A. (1964) Nicotinamide adenine dinucleotide phosphate-specific dehydrogenases in relation to lipogenesis. Biochem. Biophys. Acta 84:239-250.

Patel, T.B. and Clark, J.B. (1980) Lipogenesis in the brain of suckling rats. Biochem. J. 188:163-168.

Patton, J.S., Maxwell, M.S. and Moon, T.W. (1978) Aspects of lipid synthesis, hydrolysis, and transport studied in selected Amazon fish. Can. J. Zool. 56:787-792.

Pearce, J. (1980) Comparative aspects of lipid metabolism in avian species. Biochem. Soc. Trans. 8:295-296.

Phillips, J.W. and Hird, F.J.R. (1977a) Gluconeogenesis in vertebrate livers. Comp. Biochen. Physiol. 57B:127-131.

Phillips, J.W. and Hird, F.J.R. (1977b) Ketogenesis in vertebrate liver. Comp. Biochem. Physiol. 57B:133-138.

Phillipson, A.T. (1947) The production of fatty acids in the alimentary tract of the dog. J. Exp. Biol. 23:346-349.

Prior, R.L. and Scott, R.A. (1980) Effects of intravenous infusions of glucose, lactate, propionate or acetate on the induction of lipogenesis in bovine adipose tissue. J. Nutr. 110:2011-2019.

Raulin, J. and Grundt, I.K. (1980) Incorporation of ^{14}C from carboxyl-labeled oleoyl-, linoleoyl-, and arachidonyl-CoA into water soluble and insoluble fractions of rat liver slices: methodology for in vitro experiments. Anal. Biochem. 101:204-214.

Renaud, J.M. and Moon, T.W. (1980a) Characterization of gluconeogenesis in hepatocytes isolated from the American eel, *Anguilla rostrata* LeSueur. J. Comp. Physiol. 135:115-125.

Renaud, J.M. and Moon, T.W. (1980b) Starvation and the metabolism of hepatocytes isolated from the American eel, *A. rostrata* LeSueur. J. Comp. Physiol. 135:127-137.

Robinson, A.M. and Williamson, D.H. (1980) Physiological roles of ketone bodies as substrates and signals in mammalian tissues. Physiol. Rev. 60:143-187.

Robinson, J.S. and Mead, J.F. (1973) Lipid absorption and deposition in rainbow trout. Can. J. Biochem. 51:1050-1058.

Rogers, Q.R., Morris, J.G. and Freedland, R.A. (1977) Lack of hepatic enzymatic adaptation to low and high levels of dietary protein in the adult cat. Enzyme 22:348-356.

Romsos, D.R. and Leveille, G.A. (1974) Effect of diet on activity of enzymes involved in fatty acid and cholesterol synthesis. Adv. Lipid Res. 12:97-146.

Romsos, D.R. and Leveille, G.A. (1975) Dietary regulation of lipid metabolism. *In* Modification of Lipid Metabolism (Edited by Perkins, E.G. and Witting, L.A.), pp. 127-142. Academic Press, New York.

Rous, S. (1971) The origin of hydrogen in fatty acid synthesis. Adv. Lipid. Res. 9:73-118.

Rous, S. (1976) Relative importance of acetate, acetoacetate and D-β-OH-butyrate in the transport of acetyl CoA from the mitochondria to the cytoplasm for fatty acid synthesis in mice. Life Sciences 18:633-638.

Rous, S. (1977) Fasting and insulin regulation of the utilization of acetoacetate for fatty acid synthesis. Arch. Biochem. Biophys. 179:328-333.

Rous, S. (1978) Possible contribution of (2-^{3}H)malate and (2, 3-^{3}H) succinate tritium to the same tritiated NADPH pool for participation in fatty acid synthesis. Biochimie 60:111-117.

Rous, S. and Favarger, P. (1973) The role of acetoacetate in the transfer of acetyl units outside the mitochondria in liver and adipose tissues of rats or mice. FEBS Lett. 37:231-232.

Saggerson, E.D. (1974a) Lipogenesis in rat and guinea pig isolated epididymal fat-cells. Biochem J. 140:211-224.

Saggerson, E.D. (1974b) Lipogenesis in rabbit isolated fat-cells. Biochem J. 142:477-482.

Saggerson, E.D. (1980) Regulation of lipid metabolism in adipose tissue and liver cells. *In* Biochemistry of Cellular Regulation, Volume II, Clinical and Scientific Aspects of the Regulation of Metabolism (Edited by Ashwell, M.), pp 207-256. CRC Press, Inc. Boca Raton, Florida.

Salmon, D.M., Bowen, N.L. and Hems, D.A. (1974) Synthesis of fatty acids in the perfused mouse liver. Biochem. J. 142:611-618.

Sargent, J.R. and Henderson, J. (1980) Lipid metabolism in marine animals. Biochem. Soc. Trans. 8:296-297.

Saxena, S.C. and Zandee, D.I. (1971) Biosynthesis of lipids and fatty acids in skin and body of a fresh water carp, *Scardinius erythrophthalmus* L., after the injection of (1-^{14}C) sodium acetate. Arch. Int. Physiol. Biochim. 19:499-510.

Sera, H. and Ishida, Y. (1972) Bacterial flora in the digestive tracts of marine fish-2. Changes of bacterial flora with time lapse after ingestion of diet. Bull. Jap. Soc. Sci. Fish. 38:633-637.

Shakir, K.M.M., Sundaram, S.G. and Margolis, S. (1978) Lipid synthesis in isolated intestinal cells. J. Lipid Res. 19:433-441.

Shrago, E., Spennetta, T. and Gordon, E. (1969) Fatty acid synthesis in human adipose tissue. J. Biol. Chem. 244:2761-2766.

Shrago, E., Glennon, J.A. and Gordon, E.S. (1971) Comparative aspects of lipogenesis in mammalian tissues. Metabolism 20:54-61.

Shul'man, G.E. (1974) Life Cycles of Fish, John Wiley & Sons, Toronto.

Sinha, V.R.P. and Jones, J.W. (1975) The European Freshwater Eel, Liverpool University Press, Liverpool.

Soling, H.D., Zahlten, R., Reimold, W.V. and Willms, B. (1970) Utilization of ketone bodies by adipose tissue and its regulation by carbohydrate metabolism. Horm. Metab. Res. 2:56-61.

Spencer, A.F. and Lowenstein, J.M. (1962) The supply of precursors for the synthesis of fatty acids. J. Biol. Chem. 237:3640-3648.

Srere, P.A. (1965) The molecular physiology of citrate. Nature (London) 205:766-768.

Steel, R.G.D., and Torrie, J.H. (1960) Principles and procedures of statistics. McGraw-Hill Book Co. New York.

Stucki, J.W., Brawand, F. and Walter, P. (1972) Regulation of pyruvate metabolism in rat liver mitochondria by adenine nucleotides and fatty acids. Eur. J. Biochem. 27:181-191.

Sullivan, A.C., Hamilton, J.G., Miller, O.N. and Wheatley, V.R. (1972) Inhibition of lipogenesis in rat liver by (-)-hydroxycitrate. Arch. Biochem. Biophys. 150:183-190.

Taketa, K., Kaneshige, Y., Tanaka, A. and Kosaka, K. (1970) Differential responses of glucose-6-phosphate dehydrogenase and lipogenesis to fat-free diets. Biochem. Med. 4:531-545.

Tashima, L.S. and Cahill, G.F. Jr. (1965) Fat metabolism in fish. In Handbook of Physiology, Section 5, Adipose Tissue (Edited by Renold, A.E. and Cahill, G.F. Jr.), pp 55-58. Amer. Physiol. Soc. Washington D.C.

Tashima, L. and Cahill, G.F. Jr. (1968) Effects of insulin in the toadfish, *Opsanus tau*. Gen. Comp. Endocrinol. 11:262-271.

Tepperman, J. and Tepperman, H.M. (1970) Gluconeogenesis, lipogenesis and the Sherringtonian metapher. Federation Proc. 29:1284-1293.

Thurman, R.G. and Scholz, R. (1973) interaction of mixed-function oxidation with biosynthesis processes. Eur. J. Biochem. 38:73-78.

Ugajin, M. (1979) Studies on the taxonomy of major microflora on the intestinal contents of salmonids. Bull. Jap. Soc. Sci. Fish. 45:721-731.

Vague, J. and Fenasse, R. (1965) Comparative anatomy of adipose tissue. In Handbook of Physiology, Section 5, Adipose Tissues (Edited by Renold, A.E. and Fenasse, G.F. Jr.), pp 55-58. Amer. Physiol. Soc., Washington, D.C.

Veech, R.L., Eggleston, L.V. and Krebs, H.A. (1969) The redox state of free nicotinamide adenine dinucleotide phosphate in the cytoplasm of rat liver. Biochem. J. 115:609-619.

Vernon, A.G. (1980) Comparative aspects of lipid metabolism in monogastric, pre-ruminant and ruminating animals. Biochem. Soc. Trans. 8:291-292.

Webber, R.J. and Edmond, J. (1979) The in vivo utilization of aceto-acetate, D-(-)-3-hydroxybutyrate, and glucose for lipid synthesis in brain in the 18-day-old rat. J. Biol. Chem. 254:3912-3920.

Whitehurst, G.B., Beitz, D.C., Pothaven, M.A., Ellison, W.R. and Crump, M.H. (1978) Lactate as a percusor of fatty acids in bovine adipose tissue. J. Nutr. 108:1806-1811.

Wilkins, N.P. (1967) Starvation of the herring, *Clupea harengus* L; survival and some gross biochemical changes. Comp. Biochem. physiol. 23:503-518.

Williamson, D.H., Keown, S.R. and Ilic, V. (1975) Metabolic interactions of glucose, acetoacetate and insulin in mammary gland slices of lactating rats. Biochem. J. 150:145-152.

Wills, R.B.H. and Hopkirk, G. (1976) Distribution and fatty acid composition of lipids of eels (*Anguilla australis*). Comp. Biochem. Physiol. 53B:525-527.

Yeh, Y.Y., Streuli, V.L. and Zee, P. (1977) Relative utilization of fatty acids for synthesis of ketone bodies and complex lipids in the liver of developing rats. Lipids. 12:367-374.

Zammit, V.A., Beis, A. and Newsholme, E.A. (1979) The role of 3-oxo acid-CoA transferase in the regulation of ketogenesis in the liver. FEBS Lett. 103:212-215.

Zammit, V.A. and Newsholme, E.A. (1979) Activities of enzymes of fat and ketone-body metabolism and effects of starvation on blood concentrations of glucose and fat fuels in teleost and elasmobranch fish. Biochem. J. 184:313-322.

APPENDIX 1

The variability of measurements of in vitro lipid synthesis, saponification, and incorporation of ^3H_2O into lipid-free dry precipitates.

	Eel 1		Eel 2		Eel 3	
	Rate/fr. %	var. %	Rate/fr. %	var. %	Rate/fr. %	var. %
Lipid synthesis:						
Rate	243.6 ± 2.8	1.1%	160.7 ± 1.1	0.7%	54.2 ± 1.0	1.9%
Saponification: fraction %						
Non-saponifiables	2.36 ± 0.15	6.5%	2.48 ± 0.22	8.8%	3.11 ± 0.59	18.8%
Fatty Acids	82.25 ± 0.28	0.3%	86.64 ± 0.26	0.3%	75.48 ± 0.28	0.4%
Glycerol	15.38 ± 0.20	1.3%	10.88 ± 0.35	3.2%	21.41 ± 0.76	3.5%
Lipid-free dry weight:						
Rate	15.7 ± 1.2	7.7%	35.2 ± 1.1	3.2%	26.2 ± 1.2	4.6%

Liver slices from each eel were used for three identical incubations. All flasks contained 5 mM glucose, lactate, and acetate, and 2 mCi ^3H_2O. Incubation and subsequent procedures wer performed as described in the Materials and Methods. Rates of lipid synthesis are expressed as nmoles ^3H_2O incorporated into lipids/100 mg wet weight/2 hr at 15°C (mean±SEM). Saponification values are fraction percents (fr. %) of label incorporation (see Table 11). Rates of ^3H_2O incorporation into lipid-free precipitates are expressed as dpm x 10^{-2}/100 mg wet weight/2 hr at 15°C. Variability % (var. %) is SEM x 100/rate or %.

APPENDIX 2

The effect of added substrates on ^3H_2O incorporation into different lipid fractions.[1]

Substrate(s) + ^3H_2O	(n)	% FA	% Glycerol	% NS
Glucose + lactate	7	72.2 ± 3.9	26.4 ± 3.7	1.4 ± 0.6
None	7	54.3 ± 4.7	44.2 ± 4.5	1.5 ± 0.5
Acetate	3	59.4 ± 3.9	39.2 ± 4.5	1.4 ± 0.9
Glucose + acetate	3	60.0 ± 5.5	39.4 ± 5.4	0.6 ± 0.3
Glucose + acetoacetate	3	77.6 ± 6.4	21.3 ± 6.1	1.1 ± 0.3
Glucose + leucine	3	65.4 ± 5.0	33.9 ± 4.9	0.6 ± 0.2

[1]Tritiated water incorporation into fatty acids (FA), glycerol, or non-saponifiables (NS) x 100/tritiated water incorporation in total lipid extract (mean fraction % ± SEM). Corresponding rates of ^3H_2O incorporation are presented in Table 7 (fed animals).

APPENDIX 3

Percent of total radioactivity in various lipid classes after injection of 1-^{14}C-acetate.

	Tissue			
	Liver (n=3)	Intestine (n=4)	Plasma (n=1)	Whole Side (n=1)
Polar lipids	68.5 ± 1.2[1]	65.3 ± 2.1	61.2	73.2
Monoacylglycerols	2.2 ± 0.2	3.7 ± 1.0	2.3	4.6
Free fatty acids	1.4 ± 0.6	2.5 ± 0.4	3.5	4.0
Cholesterol	1.7 ± 0.3	3.0 ± 0.6	4.7	4.6
Diacylglycerols	8.4 ± 2.2	2.8 ± 0.5	2.3	1.1
Triacylglycerols	17.8 ± 1.1	22.7 ± 2.1	21.2	10.8
Cholesterol esters			4.7	1.7

Eels were injected with 1-^{14}C-acetate (1-2 µCi/g, specific activity 54.6 mCi/nmole) and killed 24 hr later. Animals were fed worms for 9 months prior to the experiments. [1]Mean % ± SEM.

APPENDIX 4

Weight percentages of major components in lipid extracts from eel liver.

	Weight %	
	Fed (n=2) (beef liver)	Starved (n=1) (8 mo)
Polar lipids	47.9	50.0
Monoacylglycerols	3.4	3.8
Free fatty acids	3.9	11.3
Cholesterol	3.3	2.4
Diacylglycerols	5.7	0.9
Triacylglycerols	20.9	23.1
Cholesterol esters	14.9	8.5

Lipid classes were seperated by TLC using ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2), followed by ethyl ether-hexane (6:94) (Freeman and West, 1966). The amount of lipid in each class was determined spectrophotometrically after dichromate reduction (Amenta, 1964). Procedures were performed on two fed (mean presented in Table) and one starved eel.

APPENDIX 5

Lipid content of eel tissues.

Tissue/condition	% wet weight
Beef liver-fed (n=5):	
Visceral fat[1]	9.5
Red muscle	8.6 ± 2.4
Brain	8.0 ± 0.9
Liver	5.6 ± 0.3
Skin	3.6 ± 0.7
Kidney	3.4 ± 0.3
Heart	3.0 ± 0.2
Gills	2.8 ± 0.2
Whole side	2.6 ± 0.4
Blood	2.5 ± 0.5
Spleen	2.4 ± 0.5
Stomach	2.4 ± 0.4
Intestine	2.3 ± 0.2
White muscle	1.8 ± 0.3
Liver slices:	
Worm-fed (n=34)	4.6 ± 0.4
Fasted 4 mo (n=3)	4.7 ± 0.3
Starved 9 mo (n=3)	4.4 ± 0.1
Starved 22 mo (n=1)	5.0

[1] (n=1). Subcutaneous lipid (red muscle, skin) and hepatic lipid are the major fat depots in eels of this size (approx. 100 g) based upon the % lipid (above) and weight % of each tissue (Table 2).

APPENDIX 6

Addendum

It has been brought to my attention that the proposed incorporation of hepatic acetoacetate into lipids could be considered a futile cycle. Clearly, if this substrate is important in vivo, an exogenous source of carbon other than acetoacetate must continually enter the liver, and as yet this source is unknown.