

GLUCONEOGENESIS IN ISOLATED HEPATOCYTES
OF THE AMERICAN EEL, ANGUILLA ROSTRATA LESUEUR.

by

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ABSTRACT

Gluconeogenesis has been studied in immature American eels, Anguilla rostrata LeSueur. Eels were divided into two experimental groups. One group was fed every day and held at 15°C while the other group was food deprived for 10 months and held either at 15°C or at 5°C. The latter condition (food deprived at 5°C) was considered equivalent to a natural winter fast which occurs during the immature stage of the American eel. Gluconeogenic, lipogenic, glycogenic and oxidative rates from ^{14}C -lactate, ^{14}C -alanine, ^{14}C -aspartate were estimated in the fed state and at each month of food deprivation (at both 15°C and 5°C), and from ^{14}C -glycerol only in the fed eel, using an isolated eel hepatocyte preparation. Simultaneously, glucose, lactate, amino acid, glycogen and protein levels were estimated in eel plasma, liver and white skeletal muscle.

Hepatocytes isolated from fed eels maintained membrane intactness, cell numbers, ionic composition (Na^+ , K^+ , Ca^{++}), protein content, energy charge, rates of oxygen uptake, total glucose release and gluconeogenesis from 1 mM lactate during a 2 hr incubation period. The substrates, ^{14}C -lactate, ^{14}C -glucose, ^{14}C -alanine and ^{14}C -aspartate, were incorporated into the glycerol fraction, not the fatty acid fraction, of the lipids extracted from fed eel hepatocytes incubated 1 hr at 15°C.

Incubating hepatocytes with 10 mM substrates, the following results were collected: 1) glycerol, alanine and lactate, in that order, were effective gluconeogenic substrates in fed eel hepatocytes; 2) these three substrates inhibited glucose release from glycogen stores (glycogenolysis), while aspartate, the only poor gluconeogenic substrate, had no effect; 3) high rates of glycerol production (glycerogenesis) and subsequent incorporation into lipid, were observed from lactate, alanine, and

aspartate; 4) incorporation into glycogen was low from all four substrates; and 5) alanine oxidation was 7-times higher than that observed from the other substrates.

Incubating eel hepatocytes with low or physiological substrate concentrations, the following results were collected: 1) lactate was the most effective gluconeogenic substrate in both fed and starved eel hepatocytes; 2) in fed hepatocytes, gluconeogenic rates from lactate were twice those of alanine, but during starvation lactate gluconeogenesis increased significantly and alanine gluconeogenesis generally decreased, thus differences in gluconeogenic rates between these two substrates were 30 to 70-times; 3) in most experimental conditions, aspartate gluconeogenesis was insignificant when compared to the other two substrates; 4) only at the 2nd month of starvation at 5° and 15°C and the 8th month at 15°C, did gluconeogenic rates from alanine and aspartate increase significantly; 5) glycerol production and incorporation into lipid from the three substrates increased until the 5th month at 5° and 15°C followed by a gradual decline thereafter; 6) substrate incorporation into glycogen was low during starvation, but rates increased from the 3rd to the 5th month at 15°C; 7) rates of substrate oxidation appeared sufficient to provide adequate ATP quantities to sustain gluconeogenesis in both fed and starved eel hepatocytes; 8) glucagon stimulated lactate gluconeogenesis, but not amino acid gluconeogenesis in fed and late starved eel hepatocytes; 9) glucagon had no significant effect on glycogenolytic rates in fed eel hepatocytes; and 10) cortisol increased gluconeogenic rates from 1 mM lactate in fed eel hepatocytes.

Major changes in metabolite concentrations that occurred during starvation were 1) a significant increase in plasma glucose and amino acid

levels during the first 7 months of starvation at 5° and 15°C; 2) a significant liver glycogen depletion at the 2nd month at both temperatures was followed by a return to control values at the 3rd month and a second depletion during late starvation, especially at 15°C; 3) significant protein depletion in white skeletal muscle at the 3rd month of starvation at 5° and 15°C which followed the high gluconeogenic rates observed from alanine and aspartate.

These data suggest that 1) the eel liver gluconeogenic pathway is preferrently stimulated relative to the glycogenolytic pathway to produce plasma glucose; 2) one function of eel gluconeogenesis is the recycling of glucose from lactate which is produced by anaerobic carbohydrate metabolism in white skeletal muscle; 3) a second function is the synthesis of glucose from non-carbohydrate precursors which may arise from the diet or white skeletal muscle protein, especially during starvation; 4) a third function is the synthesis of glycerol for lipogenesis; 5) the simultaneous increase of gluconeogenic and lipogenic rates during starvation is evidence for the lack of reciprocal relationship between these two pathways; and 6) the important role of lactate, alanine and aspartate for ATP production to sustain gluconeogenesis is evidence for the lack of a reciprocal relationship between pyruvate carboxylase and pyruvate dehydrogenase in eel liver mitochondria.

RESUME

La gluconéogénèse a été étudiée chez l'anguille Américaine immature, Anguilla rostrata LeSueur. Les anguilles ont été divisées en deux groupes expérimentaux, le premier nourri à chaque jour et gardé à 15°C, le second privé de nourriture durant dix mois et gardé à 15°C ou 5°C. Cette dernière condition (le jeûne à 5°C) est considérée équivalente au jeûne hivernal naturel que l'on retrouve durant la phase immature de l'anguille Américaine. Les taux d'oxydation, de gluconéogénèse de lipogénèse et de glycogénèse, à partir du ^{14}C -lactate, ^{14}C -alanine, ^{14}C -aspartate, ont été estimés chez l'anguille nourrie, et à chaque mois du jeûne à 15°C et 5°C; ainsi qu'à partir du ^{14}C -glycérol seulement chez l'anguille nourrie en utilisant une préparation d'hépatocytes isolées. Simultanément, les contenus en glucose, lactate, acides aminées, glycogène et protéine ont été estimées dans le plasma sanguin, le foie et le muscle squelettique blanc de l'anguille.

Les hépatocytes provenant d'anguilles nourries maintiennent pendant 2 hr d'incubation la membrane cellulaire intacte, le nombre initial de cellules, la composition ionique (Na^+ , K^+ , Ca^{++}), le contenu en protéine, la charge énergétique, les taux de respiration, de libération totale de glucose et de gluconéogénèse à partir de 1 mM de lactate. Les substrates, ^{14}C -lactate, ^{14}C -alanine, ^{14}C -glucose et ^{14}C -aspartate, ont été exclusivement incorporés dans la fraction glycérol des lipides extraits d'hépatocytes, provenant d'anguilles nourries, et incubées 1 hr à 15°C.

Suite à l'incubation d'hépatocytes en présence de substrats 10 mM, les résultats suivants ont été recueillis: 1) le glycérol, l'alanine et le lactate, dans cet ordre, se sont avérés les substrats gluconéogéniques les plus efficaces chez les hépatocytes provenant d'anguilles nourries;

2) ces trois substrats ont inhibé la libération du glucose à partir des réserves de glycogène (glycolyse) alors que l'aspartate, le seul faible substrat gluconéogénique ne démontra aucun effet; 3) de hauts taux de production de glycérol (glycérogénèse) et subséquemment d'incorporation dans les lipides ont été observées à partir du lactate, de l'alanine et de l'aspartate; 4) l'incorporation des substrats dans le glycogène a été très bas à partir de tous les substrats; et 5) l'oxydation de l'alanine a été 7 fois supérieur aux taux observés à partir des autres substrats.

Suite à l'incubation d'hépatocytes en présence de substrats à concentrations faibles ou physiologiques, les résultats suivants ont été recueillis: 1) le lactate est le substrat gluconéogénique le plus efficace tant chez les hépatocytes d'anguilles nourries et à jeûns; 2) chez les hépatocytes d'anguilles nourries les taux gluconéogéniques à partir du lactate ont été 2 fois ceux de l'alanine, mais durant le jeûne, la gluconéogénèse à partir du lactate augmenta significativement alors que généralement la gluconéogénèse à partir de l'alanine diminua, démontrant ainsi des différences pour les taux gluconéogéniques de l'ordre de 30 à 70 fois plus élevé entre les deux substrats; 3) pour la plupart des conditions expérimentales, la gluconéogénèse à partir de l'aspartate a été non-significative lorsque comparée aux deux autres substrats; 4) les taux gluconéogéniques à partir de l'alanine et de l'aspartate ont significativement augmenté lors du 2^{ième} mois de jeûne à 5[°] et 15[°]C et le 8^{ième} mois à 15[°]C; 5) la production à partir des trois substrats et l'incorporation du glycérol dans les lipides augmenta jusqu'au 5^{ième} mois à 5[°] et 15[°]C suivies d'une diminution graduelle par la suite; 6) l'incorporation des substrats dans le glycogène a été faible durant le jeûne, mais à 15[°]C les taux ont augmenté à partir du 3^{ième} au 5^{ième} mois; 7) les taux d'oxydation des substrats se sont avérés suffisant pour

procurer des quantités adéquates d'ATP de façon à soutenir la gluconéogénèse chez les hépatocytes d'anguilles nourries et à jeûns; 7) le glucagon a stimulé la gluconéogénèse à partir du lactate, non à partir des acides aminées, chez les hépatocytes d'anguilles nourries et durant le jeûne prolongé; 9) le glucagon ne démontra aucun effet significatif sur les taux glycogénolytique chez les hépatocytes d'anguilles nourries; 10) le cortisol a augmenté les taux gluconéogéniques à partir de 1mM de lactate chez les hépatocytes d'anguilles nourries.

Les principaux changements dans les contenus métaboliques qui ont été observés durant le jeûne sont 1) une augmentation significative des concentrations de glucose et d'acides aminées dans le plasma sanguin durant les 7 premiers mois de jeûne à 5°C et 15°C; 2) une perte significative des réserves de glycogène dans le foie au 2ième mois aux deux températures de jeûne suivi d'un retour à la normale au 3ième mois et d'une seconde perte durant le jeûne prolongé, spécialement à 15°C; et 3) une perte significative en protéine dans les muscles squelettiques blancs au 3ième mois de jeûne à 5°C et 15°C, ce qui suivait les hauts taux gluconéogéniques observés à partir de l'alanine et de l'aspartate.

Ces résultats suggèrent que 1) la voie métabolique de la gluconéogénèse dans le foie de l'anguille est de préférence augmentée relativement à la voie métabolique de la glycogénolyse pour produire du glucose sanguin; 2) une fonction de la gluconéogénèse chez l'anguille est le recyclage du glucose à partir du lactate qui est produit lors du métabolisme anaérobie du glucose dans le muscle squelettique blanc; 3) une deuxième fonction est la synthèse du glucose à partir des substrats non-glucidiques qui peuvent être obtenus soit de la nourriture ou des protéines contenues dans le muscle squelettique blanc, spécialement durant le jeûne; 4) une

troisième fonction est la synthèse du glycérol pour la lipogénèse;

5) l'augmentation simultanée des taux gluconéogéniques et lipogéniques durant le jeûne est une évidence pour le manque de relation réciproque entre ces deux voies métaboliques; et 6) le rôle important du lactate, de l'alanine et de l'aspartate dans la production de l'ATP pour soutenir la gluconéogénèse est une évidence pour l'absence de relation réciproque entre la carboxylase du pyruvate et la déshydrogénase du pyruvate dans les mitochondries du foie d'anguille.

ABBREVIATIONS

ALA T	Alanine-oxo-acid aminotransferase (EC 2.6.1.2) L-alanine: 2-oxoglutarate aminotransferase)
FDPase	Fructose diphosphatase (EC 3.1.3.11 D-Fructose-1, 6-diphosphate 1-phosphohydrolase)
G-6-Pase	Glucose-6-phosphatase (EC 3.1.3.9 D-Glucose-6-phosphate phosphohydrolase).
α GPD	α Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12 D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating)).
LDH	Lactic dehydrogenase (EC 1.1.1.27 L-lactate: NAD oxidoreductase).
PC	Pyruvate carboxylase (EC 6.4.1.1 Pyruvate: carbondioxide ligase (ADP)).
PDH	Pyruvate dehydrogenase (EC 1.2.7.1. Pyruvate: ferredoxin oxidoreductase (CoA-acetylating)).
PEP CK	Phosphoenol-pyruvate carboxykinase (EC 4.1.1.32 GTP: oxaloacetate carboxylase (transphosphorylating)).
PFK	Phosphofructokinase (EC 2.7.1.11 ATP:D-Fructose-6-phosphate 1-phosphotransferase).
PK	Pyruvate kinase (EC 2.4.1.1 ATP: Pyruvate phosphotransferase).
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	3',5' cyclic adenosine monophosphate
DHA	Dihydroxyacetone
FDP	Fructose-1, 6-diphosphate
F-6-P	Fructose-6-phosphate
G-6-P	Glucose-6-phosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
α KG	α -Ketoglutarate
MS 222	Ethyl-M-aminobenzoate methane sulfonic acid

NAD	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
OXA	Oxaloacetate
PEP	Phosphoenol pyruvate
Pi	Inorganic phosphate

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I

INTRODUCTION

INTRODUCTION

Blood glucose levels are subject to tight homeostatic control in mammals for two reasons. Firstly, a certain quantity of glucose is required for the maintenance of nerve cells, red blood cells and kidney medulla cells since glucose is their sole energy source. Secondly, if blood glucose exceeds the transport maximum of the kidney, some glucose will appear in the urine (Mudge et al., 1973). The remarkable stability of blood glucose indicates that precise regulatory mechanisms govern liver function, the major organ involved in glucose homeostasis.

The major source of glucose during adequate nutritional conditions in mammals is from ingested food and the glucose excess is stored as glycogen by the process known as glycogenesis. With food deprivation, however, blood glucose may arise from two sources: glycogenolysis and gluconeogenesis. The first, glycogenolysis, is the production of glucose from stored glycogen. The importance of glycogenolysis is limited since rat liver glycogen is spent within 48 hr of food deprivation (Steiner and Williams, 1959; Newsholme and Start, 1976).

The second source, gluconeogenesis, is the synthesis of glucose from smaller precursors such as lactate, glycerol and amino acids (Walter, 1973). This process excludes synthesis of glucose from carbohydrates such as fructose, mannose or glycogen. Thus after 48 hr of starvation, gluconeogenic rates increase in rat liver to maintain blood glucose since glycogen stores are depleted (Ross et al., 1967a,b; Johnson et al., 1972).

Blood glucose homeostasis in fish is less precise and glycogen stores may remain essentially constant for months during starvation in some fish (Stimpson, 1965; Dave et al., 1975; Ince and Thorpe, 1977a).

Even if glycogenolysis (Birnbaum et al., 1976) and gluconeogenesis (Cowey et al., 1977a,b; Phillips and Hird, 1977) are present in fish liver, their exact role with respect to glucose production during a fed or a starved state have not been demonstrated experimentally.

In this study, gluconeogenic rates are estimated in hepatocytes prepared from the liver of the American eel, Anguilla rostrata LeSueur. The importance of gluconeogenesis as a source of blood glucose is studied in fed (Chapter 3) and food deprived (Chapter 4) eels. In this Introduction, gluconeogenesis is reviewed in terms of its pathway, control and major functions based upon the extensive literature on the rat liver. Also, fish energy metabolism will be reviewed to define the metabolic function of glucose and ultimately the role of gluconeogenesis in fish compared to mammals.

THE GLUCONEOGENIC PATHWAY

The gluconeogenic pathway in rat liver is well documented (see reviews by Eisenstein, 1967; Walter, 1973). Beginning with lactate, the gluconeogenic pathway is essentially the reverse of the glycolytic pathway since many enzymic steps are reversible (Fig. 1). Due to large energy changes, however, three irreversible steps exist within glycolysis: glucose to glucose-6-phosphate (G-6-P) conversion by hexokinase (HK), fructose-6-phosphate (F-6-P) to fructose-1,6-diphosphate (FDP) conversion by phosphofructokinase (PFK), and phosphoenol pyruvate (PEP) to pyruvate conversion by pyruvate kinase (PK). During gluconeogenesis, the first two steps are circumvented by glucose-6-phosphatase (G-6-Pase) and fructose diphosphatase (FDPase), respectively. The bypass of PK involves two enzymes: phosphoenol pyruvate carboxykinase (PEP CK) and pyruvate

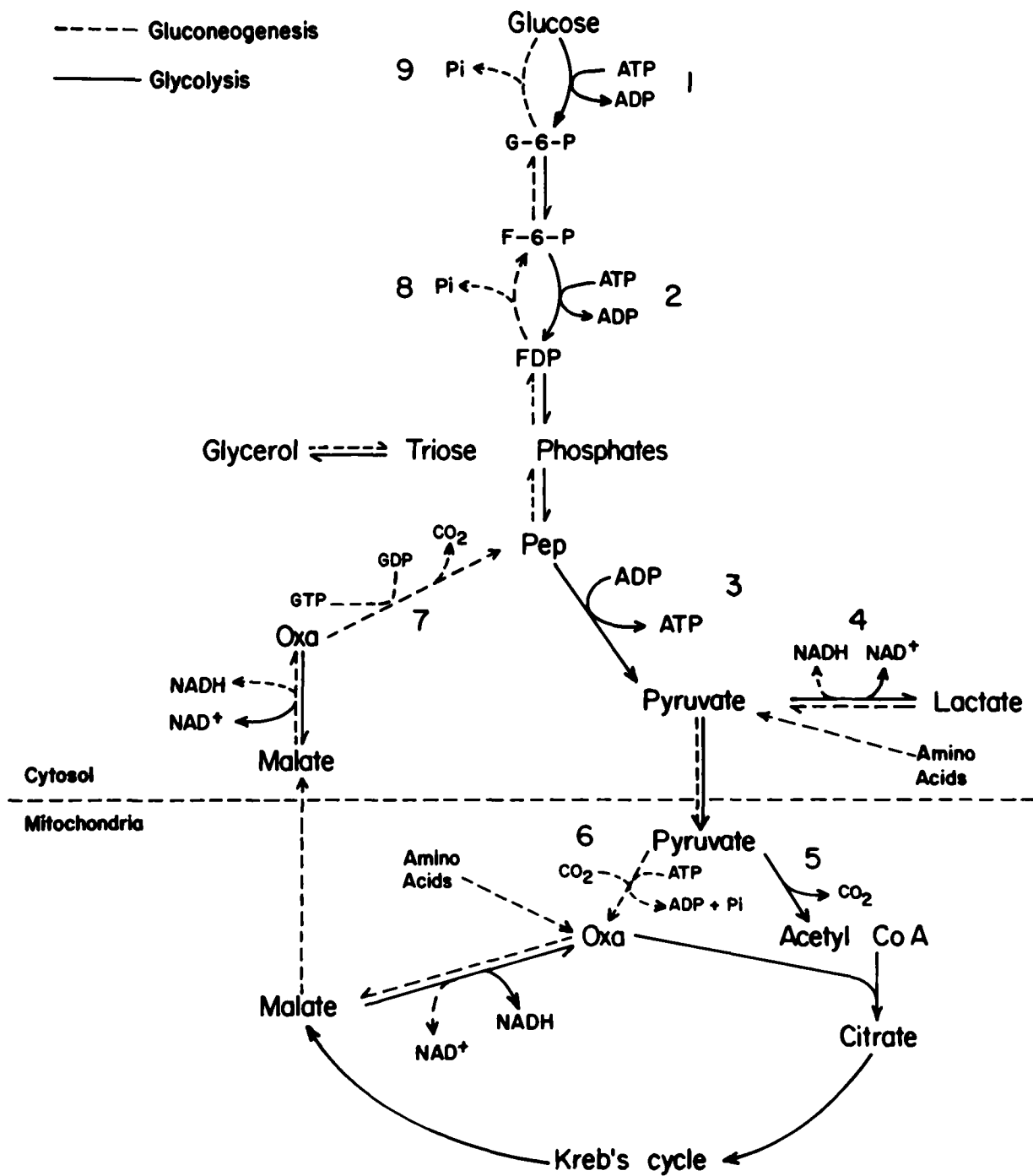


Figure 1. Gluconeogenic pathway in rat liver. Glycolytic enzymes:

- 1) Hexokinase (HK); 2) Phosphofructokinase (PFK);
- 3) Pyruvate kinase (PK); 4) Lactate dehydrogenase (LDH);
- 5) Pyruvate dehydrogenase (PDH). Gluconeogenic enzymes:
- 6) Pyruvate carboxylase (PC); 7) Phosphoenol pyruvate carboxykinase (PEP CK); 8) Fructose-1,6-diphosphatase (FDPase); 9) Glucose-6-phosphatase (G-6-Pase). See the list of abbreviations for other terms.

carboxylase (PC). This bypass also permits the translocation of one mole of NADH^{*} per mole of pyruvate from the mitochondria to the cytosol via malate transport since PC is mitochondrial and PEP CK is usually cytosolic. This NADH is required by the enzyme glyceraldehyde-3-phosphate dehydrogenase in the gluconeogenic pathway.

CONTROL OF GLUCONEOGENESIS

The existence of these three bypass steps demands integration between gluconeogenesis and glycolysis to prevent futile cycling (Katz and Rognstad, 1976). For example, if both HK and G-6-Pase were concurrently active, the net result would be the hydrolysis of 1 mole ATP per mole of glucose. The control of these two processes (glycolysis and gluconeogenesis) is achieved at different levels, including substrate availability, cellular energy content (levels of ATP and GTP), cytosolic reducing equivalents (NAD/NADH ratio), and glucose demand.

A) Substrate availability

Gluconeogenic rates are extremely low in rat liver without exogenous substrate (Ross et al., 1967a,b; Johnson et al., 1972). A continuous supply of substrates from peripheral organs must be provided and most investigators agree that skeletal muscle is the most important organ, and muscle lactate and amino acids are the most important gluconeogenic substrates. Blood lactate concentrations increase either as the load or as the time of muscular work increases (Hermansen and Vaage, 1977), and amino acids, especially alanine, are continuously released by skeletal muscle during rest or exercise (Felig, 1973; Garber et al., 1976a).

* See list of abbreviations.

Gluconeogenic rates increase in rat liver concomitantly with increases in lactate (Exton et al., 1970; Garrison and Haynes, 1973) and amino acids, especially alanine (Mallette et al., 1969; Exton et al., 1970). Thus, substrate availability represents a potentially important integrative parameter in the control of rat liver gluconeogenesis.

B) Energy charge and redox balance

One of the most important factors in the integration between gluconeogenesis and glycolysis is cellular energy charge. This concept, first suggested by Atkinson (1977), equates the equilibrium relationship between the cellular adenylates (ATP, ADP and AMP) to their importance as modulators of enzyme activities. An example of adenylate action is the partitioning of pyruvate between PC and pyruvate dehydrogenase (PDH) in the mitochondria (See Fig. 1). The activity of PDH is inversely proportional to the ATP/ADP ratio or the energy charge (Walter et al., 1973; Siess and Wieland, 1976), while the activity of PC varies directly with the ATP/ADP ratio or the energy charge (Stucki et al., 1972; Walter et al., 1973). Thus, the activities of these two enzymes are reciprocal and as mitochondrial ATP levels rise, activities of PDH decrease and PC increase leading to oxaloacetate production and ultimately glucose production (i.e., gluconeogenesis). It should be noted that PC has an absolute requirement for the cofactor AcetylCoA (McLure et al., 1971a,b) so regulation is more complex than a simple increase in ATP. As with PC, GTP is required in the reaction catalysed by PEP CK, the second enzyme in the bypass sequence (Utter et al., 1974; Chang and Lane, 1966). Thus, an increase in GTP and/or ATP will coordinately activate both enzymes and gluconeogenesis will be favoured over glycolysis.

While activities of the gluconeogenic enzymes PEP CK and PC

increase, the activity of the corresponding glycolytic enzyme PK must decrease to prevent recycling of pyruvate from PEP. Both ATP and alanine inhibit PK by increasing the Michaelis constant for PEP, or the $K_m(\text{PEP})$, while ADP increases its activity (van Berkel, 1974). Similarly, activities of HK and PFK are inhibited by high ATP concentrations while ADP and AMP activate PFK and inhibit FDPase (Newsholme and Start, 1976). Again, it is apparent that energy charge is an important variable to prevent substrate cycling and to direct carbon flow to either the glycolytic or gluconeogenic pathways.

Similar to energy charge, the cytosolic redox balance has been implicated in the regulation of gluconeogenesis. NADH is a substrate for the enzyme glyceraldehyde-3-phosphate dehydrogenase. Since this enzyme catalyses a reversible reaction (Axelrod, 1967), as NADH concentrations increase, the more the gluconeogenic direction will be favoured, and vice versa.

It has been demonstrated that fatty acids stimulate gluconeogenesis from lactate and pyruvate in the rat liver (Johnson et al., 1972; Zaleski and Bryla, 1977). Moreover, during starvation or in the presence of glucagon, fats are mobilized and ketone bodies accumulate (Frohlich and Wieland, 1964; Zaleski and Bryla, 1977). Thus, many investigators propose that fatty acid oxidation may provide directly the ATP and indirectly the reducing equivalents (NADH) necessary for gluconeogenesis.

Therefore, a high energy charge and a high NADH/NAD ratio tend to stimulate gluconeogenesis and inhibit glycolysis, and vice versa. The former control acts primarily by modulating enzyme activities (ATP inhibits glycolytic enzymes and stimulates gluconeogenic enzymes; ADP and AMP have a reverse effect) while the latter control acts on the

equilibrium of glyceraldehyde-3-phosphate dehydrogenase (NADH favours the gluconeogenic direction).

C) Hormonal control

When glucose demand increases, eg., during starvation and hypoglycemia, plasma glucose is maintained through liver glycogenolysis and/or gluconeogenesis (Eisenstein, 1967; Walter, 1973). Hormonal changes influence the glycogenolytic and gluconeogenic rates by altering specific enzyme activities. The peptide hormones glucagon and epinephrine and the corticosteroid cortisol have been implicated in this process. Glucagon and epinephrine do not penetrate the cell membrane but interacts with specific membrane receptors to alter concentrations of the second messenger, cyclic AMP (cAMP) (Byus et al., 1976; Birnbaum and Fain, 1977). cAMP generates a cascade of enzyme phosphorylations which ultimately activates glycogen phosphorylase and deactivates glycogen synthetase to stimulate glycogenolysis (Birnbaum and Fain, 1977; Massague and Guinovart, 1977). The importance of glycogenolysis during starvation or hypoglycemia, however, is limited since in the rat liver glycogen is spent within 48 hr of food deprivation (Steiner and Williams, 1959; Newsholme and Start, 1976). Therefore, gluconeogenesis must be considered of major importance for glucose homeostasis.

The glycolytic enzymes, PFK (Wimhurst et al., 1974) and PK (Clark, 1976; Riou et al., 1976; Rognstad, 1976) are inhibited by the same cAMP-dependent mechanism described above, but there is no evidence to this point that the gluconeogenic enzymes, G-6-Pase and FDPase (Lardy et al., 1973; Pilkis et al., 1976), PEP CK and PC (Wimhurst et al., 1974) are directly activated by this mechanism even though activities increase simultaneously with gluconeogenesis. It has been reported,

however, that cAMP activates mRNA translation to increase PEP CK synthesis (Krone et al., 1976). Therefore, glucagon and epinephrine, functioning through cAMP, activate glycogenolysis, inhibits glycolysis, and indirectly activates gluconeogenesis.

The corticosteroid cortisol activates gluconeogenesis and glycogenesis in rat liver. Unlike glucagon and epinephrine, however, cortisol penetrates the cell membrane and together with a cytosolic receptor protein acts directly on nuclear DNA (Baulieu, 1975). Its major effect is to stimulate specific DNA transcription leading to increased synthesis of the gluconeogenic enzymes PEP CK, G-6-Pase, FDPase and amino acid transaminases (Eisenstein, 1967; Krone et al., 1976). In hypophysectomized or adrenalectomized rats, glucagon is not as effective as in intact rats, suggesting that cortisol plays a permissive role to glucagon by increasing DNA transcription while cAMP increases mRNA translation (Krone et al., 1975).

The peptide hormone insulin, the antagonist of glucagon, is also involved in blood glucose homeostasis. Insulin decreases blood glucose during hyperglycemia (e.g., during food ingestion) through the following actions: an increase in glucose uptake by liver and muscle (Berger et al., 1976); an increase in liver and muscle glycolysis and oxidative metabolism (Wimhurst et al., 1974; Berger et al., 1976); an increase in liver glycogenesis (Massague and Guinovart, 1977), and a decrease in liver cAMP (Exton et al., 1971; Seitz et al., 1977) which ultimately suppresses both glycogenolysis (Hers et al., 1973; Massague and Guinovart, 1977) and gluconeogenesis (Eisenstein, 1967; Exton et al., 1971; Wimhurst et al., 1974).

A last effect of these hormones is on substrate availability which

must increase to stimulate gluconeogenesis. Glucagon and cortisol increase both the quantity of amino acids released from peripheral organs and their uptake by liver cells, while insulin opposes these effects (Waterlow, 1968; Felig and Wharen, 1974).

Thus, gluconeogenic rates from lactate and amino acids are increased in rat liver if the substrate availability, energy charge, reducing equivalent and the key gluconeogenic enzyme activities increase while the key glycolytic enzyme activities decrease within the liver cells. These concerted actions are achieved through glucagon, epinephrine and cortisol, while insulin opposes these effects.

IMPORTANCE OF GLUCONEOGENESIS IN THE MAMMALIAN LIVER

Two major functions have been ascribed to gluconeogenesis: the recovery of post-exercise blood lactate and the maintenance of blood glucose levels during the post-absorptive state and under starvation conditions. Anaerobic muscle contraction produces large amounts of lactate at the expense of stored glycogen and during the recovery period blood lactate concentration initially rises sharply then decreases as glycogen stores are replenished (Hermansen and Vaage, 1977). Many years ago, it was shown by arterio-femoral vein and portal-hepatic vein differences that lactate is released by the muscle and absorbed by the liver and that glucose follows a reverse pattern (Himwich et al., 1929). From these experiments and others, the Cori cycle was proposed: lactate produced in skeletal muscle during exercise is transported by the blood to the liver where it is reconverted to glucose by gluconeogenesis. This glucose is transported back to skeletal muscle to be stored as muscle glycogen (Cori and Cori, 1929; Himwich et al., 1929).

Much experimental evidence favors the functioning of this Cori cycle. For example, activities of FDPase and PEP CK are 50-times less active in vertebrate red and white muscle than in liver, while no G-6-Pase or PC activities are detected suggesting that vertebrate skeletal muscle has a poor gluconeogenic capacity (Krebs and Woodford, 1965; Opie and Newsholme, 1967). Also, lactate is a good gluconeogenic substrate in rat liver (Johnson et al., 1972; Garrison and Haynes, 1973). Thus, all evidence indicates that as blood lactate concentrations rise after exercise, gluconeogenic rates from lactate concomittantly increase in the liver.

The importance of the Cori cycle to lactate disposal has been the subject of intense study and recent evidence suggests that its role may have been overestimated. Hermansen and Vaage (1977) have demonstrated that lactate efflux accounts for less than 10% of the lactate which disappears from skeletal muscle during a 30 min recovery period following heavy exercise while glucose uptake from the blood by the same muscle could account for only about 3% of the muscle glycogen synthesis during the same period. Even though PC cannot be detected in mammalian muscle, these investigators felt that lactate conversion to glycogen is theoretically possible since lactate was the only metabolite present in a concentration high enough to account for the large and rapid synthesis of glycogen, i.e., they excluded the role of the liver in the replenishment of glycogen muscle.

The importance of the Cori cycle has also been estimated in vivo (Fremenet and Poyart, 1975). Approximately 71% of lactate turnover was from glucose. Moreover, the total glucose recycling rate was about $3.7 \text{ mg min}^{-1} \text{ Kg}^{0.75}$ and the Cori cycle represents only 20-30% of this

rate.

These estimates of the Cori cycle have been obtained in fed rats in a resting state, but these percentages could be altered during an exercise recovery period or starvation. For example, Owen et al. (1969) demonstrated that during prolonged starvation in man (5-6 weeks), half of the glucose produced by gluconeogenesis was from lactate and pyruvate. Nevertheless, most investigators agree that approximately 70% of the total glucose recycled must be derived from substrates other than lactate in the fed rat.

It has been proposed by Felig (1973) that a fraction of this 70% may come from the "glucose-alanine cycle". Alanine is produced from glucose in the muscle, released during exercise, transported by the blood to the liver where glucose synthesis via gluconeogenesis occurs, and the glucose is then transported back to the muscle. The evidence of this cycle is: 1) alanine release by skeletal muscle constitutes 30% of the total amino acids released; 2) increases in alanine output are proportional to the exercise intensity; 3) alanine release is stimulated by the addition of pyruvate and ammonium chloride but inhibited by aminooxyacetic acid, an inhibitor of alanine amino transferase (ALA T) suggesting alanine is derived de novo from pyruvate (Felig, 1973; Felig and Wharren, 1974; Garber et al., 1976 a); 4) alanine is rapidly accumulated by liver cells, and in the eviscerated nephrectomized rat, plasma alanine is 6-times higher than normal (Aikawa et al., 1973; and 5) alanine is rapidly converted to glucose by liver gluconeogenesis (Mallette et al., 1969; Exton et al., 1970).

Recently, Garber et al. (1976a) demonstrated no correlation between glucose uptake and amino acids release by mammalian skeletal muscle, and

that iodoacetate, a glycolytic inhibitor, prevents lactate production but not alanine release. These results led Garber and coworkers to propose that alanine is released from specific amino acids (aspartate, cysteine, leucine, valine, methionine, isoleucine, serine, threonine, and glycine) and not glucose (Garber et al., 1976b). It is probable that a "glucose-alanine cycle" as proposed originally by Felig (1973) is of no consequence in terms of glucose production; however, it is extremely important during starvation to link muscle proteolysis to amino acid gluconeogenesis in the liver, which is the second major function of gluconeogenesis.

Even though the Cori cycle may become more important during fasting (Owen et al., 1969), the net result of this cycle is only the recycling of glucose, not a net synthesis. To provide for net synthesis, as is necessary during starvation, a non-carbohydrate substrate source is required. This source is the amino acids released by skeletal muscle proteolysis. These amino acids are converted to alanine (or glutamine) by active amino transferases in skeletal muscle before being released into the blood (Garber et al., 1976b). Alanine is transported to the liver where gluconeogenic rates from alanine increase during starvation (Ross et al., 1967; Mallette et al., 1969). Finally, it has been demonstrated that alanine release by skeletal muscle was 65% higher in fasted than in fed rats, and 145% higher in the presence of added cortisone (Karl et al., 1976).

Gluconeogenesis has also been demonstrated in rat kidney cortex cells (MacDonald and Saggerson, 1977). Under normal conditions, the human kidney provides less than 10% of the total glucose produced. During prolonged starvation (5-6 weeks), the kidney provides up to one-

half of the 86 g glucose produced per day, using the same substrates as liver, but preferring amino acids (especially glutamine), glycerol, lactate and pyruvate, in that order (Owen et al., 1969). Of all the amino acids, glutamine is probably the major substrate as suggested by the following evidence: 1) glutamine is released by skeletal muscle at a rate equivalent to alanine (Garber et al., 1976a); 2) glutamine is the end-product of cysteine, leucine, valine, methionine, isoleucine, tyrosine, lysine and phenylalanine metabolism in skeletal muscle (Garber et al., 1976b); and 3) glutamine is rapidly accumulated by the kidney (Matsuke et al., 1973). The only contrary evidence of the importance of glutamine is that cortisol or fasting stimulates a redirection of carbon flow from glutamine towards alanine (Karl et al., 1976), which ultimately decreases glutamine availability to the kidney cortex cells.

In summary, gluconeogenesis has two major functions in mammals: the recovery of post-exercise blood lactate and the maintenance of blood glucose. In the fed condition, the recycling of glucose from lactate is low. In a fasting conditions, however, the rate of glucose recycling from lactate increases and and net glucose synthesis in liver from alanine and in kidney from glutamine also increases. Both alanine and glutamine are the end-products of amino acid transamination in skeletal muscle which represents a large reservoir of gluconeogenic amino acids.

Based upon limited experimental evidence investigators have generalized the gluconeogenic pattern described above, for mammals to all vertebrates, including fish (see reviews by Woodhead, 1975 (fish); Miller, 1961 (amphibian and reptile)). As will be discussed, numerous differences exist between fish and mammals with respect to life cycle, dietary composition, and changes in metabolite concentrations during

starvation. Also, the importance of glucose for the maintenance of nerve cells, red blood cells and kidney cells has yet to be experimentally defined as in mammals, and glucose metabolism in skeletal muscle is still a controversial issue (See Bilinski, 1974). Therefore, it is premature to equate fish gluconeogenesis to that seen in mammals in the absence of reliable experimental data. In the following sections, the available metabolic informations on fish will be reviewed in an attempt to find consistencies and develop a generalized metabolic pattern for fish.

GLUCONEOGENESIS IN FISH

The natural diet of most omnivores, such as the rat, probably contains adequate carbohydrate to satisfy the needs of the animal; therefore, under normal nutritional conditions, liver gluconeogenesis is probably low. The natural diet of most carnivores, including most species of fish, contains little carbohydrate. Fish require a higher dietary protein and essential amino acid intake than mammals and, growth is better on a high protein diet than on a high carbohydrate diet (Cowey, 1975). For example, when rainbow trout, Salmo gairdneri, are fed a high protein diet, they gained about 40 gm of weight in four weeks while trout fed a high carbohydrate diet gained only 5 gm during the same period (Cowey et al., 1977a).

Even though fish have a high protein requirement, it has been demonstrated that plaice, Pleuronectes platissa, fed a high protein diet containing both carbohydrate and lipid have a higher growth rate, protein efficiency (PER) and net protein utilization (NPU) than plaice fed a high protein diet containing no carbohydrate and/or no lipid;

i.e., additions of both carbohydrate and lipid to a high protein diet increase the utilization of protein for growth (PER) and decreased protein catabolism (NPU) (Cowey et al., 1975).

Similar effects of lipid and carbohydrate were observed in other fish, including the rainbow trout (Lee and Putman, 1973) and the Chinook salmon, Oncorhynchus tshawytscha (Buhler and Halver, 1961). Thus, as with the omnivorous mammals, the presence of carbohydrate and lipid as an energy source may have a beneficial effect for the carnivorous fish, but the precise role of these components is not understood.

Glucose utilization, however, is extremely low in plaice and in most fish. Eighteen hr after the intraperitoneal injection of plaice with [U-¹⁴C]-glucose, only 12% to 23% (depending upon diet) had been expired as CO₂ compared to over 30% in the mouse 1 hr after injection and 82.5% after 8 hr (Cowey et al., 1975).

Thus, the low carbohydrate diet of carnivorous fish could suggest a low glucose demand or, conversely, high gluconeogenic capacity. The former possibility is suggested by the low glucose utilization and the necessity of the latter by the effect of carbohydrates in the diet which probably decrease protein catabolism and thus increase protein utilization (PER and NPU values) for growth.

In light of these different dietary requirements in fish and mammals, it is necessary to examine the general energy metabolism of fish. This subject has been reviewed by several authors (see e.g., Love, 1970; Bilinski, 1974; Woodhead, 1975).

A) Energy metabolism in skeletal muscle

The oxidation of glycogen and long chain fatty acids are the two main energy sources for muscular work in mammals (see review by

Holloszy and Booth, 1976). Generally speaking, fatty acids predominate during prolonged periods of moderate exercise, whereas carbohydrates are used extensively in the earlier stages of the effort and during intense anaerobic exercise bursts.

Patterns of glycogen utilization during intense muscular effort in fish are basically similar to those reported for mammals (Bilinski, 1974); i.e., glycogen is depleted and lactate accumulates in muscle. Interestingly, liver glycogen and blood glucose remain constant under these conditions. Plasma lactate values increase during and after the exercise, but fish require at least 12 hr to reduce plasma lactate to resting levels unlike mammals where 30 min suffice. It has been shown, however, that this difference is principally due to a low lactate diffusion at low temperature (Black et al., 1961).

The utilization of carbohydrate and fat by fish during sustained swimming is less clear than in the case of vigorous swimming. Bilinski (1974), has recently reviewed this literature. In general as the swimming velocity increases, respiration rates tend to reach a maximum suggesting muscle energy metabolism passes from an aerobic to an anaerobic state. At low cruising speeds, glycogen depletion is observed in liver and red muscle, but not in white muscle; fat mobilization occurs only in red muscle. If high swimming speeds are maintained for 8 min, glycogen is depleted only in the white muscle.

These data are explained by the biochemical properties of white and red skeletal muscle (see Prosser, 1973). White muscle is characterized by a poor vascularity, few mitochondria per fiber, high glycogen and low lipid contents, high shortening speeds and an active anaerobic glycolytic metabolism. Red muscle, conversely, has extensive vascularity, numerous

mitochondria per fiber, high glycogen and lipid content, low shortening speeds and an aerobic metabolism which completely oxidizes fats and carbohydrates to CO_2 and H_2O . These characteristics are consistent with white muscle activities predominating at high swimming speeds, while red muscle participates at low speeds. Thus, Bilinski (1974) has suggested that fats are the primary energy substrate at low speeds and carbohydrate mobilization would increase as swimming velocities increase.

Two important conclusions arise from these studies. Firstly, carbohydrate is metabolized by skeletal muscle and at high swimming speeds it becomes the major energy source; i.e., a continuous supply of glucose is required by fish skeletal muscle since glycogen levels are typically quite low (Dave et al., 1975). Secondly, lactate is produced from carbohydrate activation in fish from skeletal muscle which could provide substrate for the Cori cycle. Thus, skeletal muscle could represent an important source of lactate for liver gluconeogenesis.

B) Energy requirement during food deprivation and migration

Two natural starvation periods are recognized in fish: a seasonal low temperature fast and a non-feeding extensive spawning migration. The former starvation condition is induced by decreases in ambient temperature and/or food availability and activity levels fall to near zero. Examples of this type are seen in the American eel, Anguilla rostrata (Vladykov, 1955), the European eel, A. anguilla (Dave et al., 1975) and the carp, Cyprinus carpio (Creach and Serfaty, 1974). Beamish (1964) showed that O_2 uptake by brook trout and white sucker decreases rapidly within the first five days of starvation, followed by a constant O_2 uptake rate for another 25 days of starvation. Also, Creach and Murat (1974) showed that production of CO_2 by starved carp from

14 C-glucose and 14 C-glucose is half of that observed in fed carp. Thus, energy demands are probably minimal at low temperatures and during starvation, and since activity is equally low, energy is required principally for the maintenance of tissue integrity. Migratory starvation, however, is associated with extensive swimming and growth of reproductive tissue, both of which require the expenditure of large amounts of energy. Examples of this type are seen in the American and European eels (Vladykov, 1955) and the king salmon, Oncorhynchus tshawytscha (Woodhead, 1975). The ultimate source of energy for both starvation types is the body reserves of carbohydrate, lipid and protein accumulated during the growing stage (see review by Woodhead, 1975).

Whereas carbohydrates play an important role in energy metabolism during muscular activity, their role during starvation remains controversial. For example, during food deprivation at low temperatures (winter starvation), severe loss of protein is observed in liver, muscle, kidney, intestine and heart in the carp (Creach and Serfaty, 1974). In the Northern pike, Esox lucius, protein content remains constant during three months of starvation while significant depletion of lipids is noted in liver and muscle after only one month of starvation, liver glycogen and blood glucose decrease after the first month of food deprivation (Ince and Thorpe, 1976a). In the golfish, Carassius auratus, lipid and protein are the main energy sources while the glycogen content of the liver is unchanged after 25 days of food deprivation (Stimpson, 1965).

Migration, as opposed to food deprivation, follows a similar pattern. Migration is associated with the maintenance of blood glucose concentrations in the Atlantic and Pacific salmons (Jonas and MacLeod, 1959). At

the end of migration, liver and muscle glycogen contents are essentially identical to pre-migratory values in the Atlantic salmon, Salmo salar (Fontaine and Hatey, 1953), while liver glycogen of the Pacific salmon, O. nerka, doubles (Chang and Idler, 1960). Liver glycogen is three times lower at the end of the migration compared to the beginning in the lamprey, Lampetra fluviatilis, but no change is observed in the glycogen content of skeletal muscle (Savina and Woztczak, 1977). Carbohydrate reserves of the Atlantic cod (Gadus morhua) are thought to be below the amount necessary to sustain migration (Kamra, 1966).

While carbohydrate reserves remain relatively constant during migration in many fish, severe loss of fat and protein is generally observed. In salmonid species, such as O. tschawytscha, fats constitute about 30% of the body weight, but this drops to 1 or 2% at the end of migration indicating a total depletion of body fat (Idler and Bitners, 1958a,b). Similar observations are reported for the coastal capelin, Mallotus villosus (Winters, 1970) and many species of cod (Damberg, 1964; Jangaard and Power, 1966). Severe loss of body protein has been reported; e.g., the salmon, O. tschawytscha uses about 30% of its body protein during migration (Idler and Bitners, 1958a,b).

Even though glucose is metabolized by skeletal muscle during muscular contraction, carbohydrate does not appear to be a major source of energy during starvation, either with food deprivation or migration. During these periods, blood glucose is maintained and liver glycogen stores are either too low or remain unchanged and, therefore, are not a good source of blood glucose. Body protein and fat, however, are rapidly depleted and appear as the major source of energy. This apparent low carbohydrate utilization could, however, be due to either

a low glucose utilization (Cowey et al., 1975; Cowey et al., 1977a) or an increase in gluconeogenesis from amino acids derived from skeletal muscle proteolysis (Stimpson, 1965; Butler, 1968; Larsson and Lewander, 1973; Dave et al., 1975).

C) Role of gluconeogenesis in fish

The presence of gluconeogenesis has been examined in fish. Phillips and Hird (1977) presented data indicating that gluconeogenic rates from radioactive lactate are 5-times higher in eel liver than rat liver slices even though the experiments were carried out at their respective body temperatures. Gluconeogenesis has also been demonstrated in vitro in homogenates or slices of lamprey liver (Phillips and Hird, 1977), trout liver (Cowey et al., 1977a) and lamprey skeletal muscle (Savina and Woztczak, 1977). Finally, the key gluconeogenic enzymes FDPase, PEP CK and PC have been demonstrated in homogenate of eel and lamprey livers (Phillips and Hird, 1977) and trout liver (Cowey et al., 1977b) and American eel liver (Hulbert, 1976; unpublished values).

The endocrine control of fish glucose metabolism has been studied but still remains obscure. Insulin plays a minor role in fish glucose homeostasis since glucose tolerance has been reported to be low in the rainbow trout (Palmer and Ryman, 1972; Cowey et al., 1977a,b) and in goldfish (Palmer and Ryman, 1972). This intolerance to glucose results from a lack of insulin release by glucose, rather than a lack of insulin effect since exogenous insulin does promote hypoglycemia in the rainbow trout (Palmer and Ryman, 1972; Cowey et al., 1977a,b) and in the European eel (Lewander et al., 1976); it also decreases alanine gluconeogenesis and PEP CK activities in trout liver (Cowey et al., 1976b). These effects of injected insulin in fish are similar to those reported

in mammals (Eisenstein, 1967; Johnson et al., 1972).

On the other hand, glucagon increases plasma glucose but has no effect on plasma free fatty acids in the European eel (Larson and Lewander, 1972) and in the carp, C. carpio (Murat et al., 1978). As with the rat hepatocyte, epinephrine increases cAMP levels and glycogenolytic rates in the goldfish hepatocytes (Brinhaum et al., 1976). In vivo, epinephrine has both hyperglycemic and lipolytic activities in the European eel following injection (Larsson, 1973) and cortisol increases liver and muscle glycogen in both the eel and the goldfish (Butler et al., 1969; Woodhead, 1975).

Generally speaking, it seems that fish liver gluconeogenesis, in terms of pathway and hormonal control, is similar to mammalian liver gluconeogenesis, and many investigators have suggested an important role of gluconeogenesis during normal nutritional conditions and/or starvation. Cowey et al. (1977a) have proposed that gluconeogenesis from amino acids is high in rainbow trout because of a naturally high protein diet, and labelled alanine injection lead rapidly to labelled plasma glucose. In the Atlantic cod, Gadus morhua, it is believed that gluconeogenesis increases during food deprivation and migration because 1) plasma pH increases under these conditions due principally to an increase in plasma amino acids and 2) liver glycogen stores are too low to maintain blood glucose over long periods of starvation (Kamra, 1966). A possible link between corticosteroid regulation of protein catabolism and gluconeogenesis in order to maintain carbohydrate levels in fish has been proposed by many authors (Butler, 1968; Woodhead, 1975; Cowey et al., 1977a).

The above studies of fish gluconeogenesis have failed to adequately

quantify gluconeogenic rates from potential substrates or the major pathway of gluconeogenesis in fish. Moreover, the concept of fish gluconeogenesis has assumed equivalence to the mammalian situation; i.e., during food deprivation or hypoglycemia, skeletal muscle proteolysis increases and the amino acids released into the blood are used for liver gluconeogenesis (Eisenstein, 1967; Felig, 1973). Even though the protein depletion observed in fish skeletal muscle may indicate a similar pattern, there are many differences between fish and mammals that must be considered before accepting this conclusion: 1) the natural fish diet contains much less carbohydrate than the normal mammalian diet and thus, fish could be adapted to lower carbohydrate levels; 2) glucose utilization in fish is about 20-30 times lower than that of mammals; 3) in mammals, glycogen is the initial source of blood glucose until its depletion after 48 hr of starvation while in most fish, glycogen stores are intact after months of starvation; and 4) in mammals, amino acid gluconeogenesis increases in the liver only after glycogen depletion. If similar gluconeogenic increases occur in fish, why are carbohydrate reserves not depleted first as in mammals? Are there advantages of keeping these reserves intact?

Presently, there is no evidence to answer these questions. Two hypotheses could, however, be proposed: 1) with food deprivation, fat and protein represent the major energy fuels and not glucose as a result of its low utilization, and thus glycogen stores are not depleted and gluconeogenic rates from amino acids do not increase significantly; or 2) glucose is an important energy source, but glycogen reserves are not depleted since gluconeogenic rates from amino

acids increase.

The purpose of this study is to define experimentally the role of gluconeogenesis in fish. The American eel, Anguilla rostrata, is an ideal system for such studies since their life cycle contains low temperature fasts and a non-feeding extensive spawning migration.

THE EEL, ANGUILLA SPP.

The natural history of eel species has been extensively studied (see reviews by Vladykov, 1955; Sinha and Jones, 1975). The spawning area for both the American eel, Anguilla rostrata, and the European eel, Anguilla anguilla, is located in an area encompassed by the Sargasso Sea. Once hatched, the eel larvae or Leptocephali grow rapidly in the sea to a glass eel and enter the freshwater rivers of North America one year later as pigmented elvers. During its upstream migration, the elver matures to a Yellow eel and grows for an additional 7 to 12 years in freshwater. A similar process occurs for the European species, but the metamorphosis in sea water requires three years. At the end of the growth period, sexual differentiation and maturation begin and many metabolic and physiological changes occur (Lewander et al., 1974). At this stage the fish is known as the Silver or Bronze eel, due to skin coloration and this stage is associated with a migration back to its ocean spawning area.

Both species of eel are subjected to natural starvation periods during winter and probably also during the spawning migration. The first starvation type occurs during the growing stage of the yellow eel when water temperatures drop and the eel stops feeding, which suggests that temperature is the trigger for cessation of eating

(Dave et al., 1975). Mayerle and Butler (1971) have shown that eels starved at 5°C had higher blood glucose and lower blood lactate levels than those starved at 15°C. Eel activities are reduced to nearly zero during the low temperature fast by burrowing into the river bottom mud to minimize energy demands required for the maintenance of tissue integrity (Nyman, 1972; Sinha and Jones, 1975).

The effects of food deprivation have been studied in the European eel (Larson and Lewander, 1973; Dave et al., 1975). During the first three months of starvation, liver and skeletal muscle fats are significantly mobilized while blood glucose and glycogen contents of both liver and muscle remain constant. Not until the third month of starvation is there significant loss of liver glycogen. Protein concentrations in liver and plasma remain relatively constant while a continuous, but insignificant decrease in skeletal muscle protein is observed during five months of starvation. Body weight loss is linear and represents 30% of the initial body weight by the end of 5 months of starvation. Therefore, even though some metabolite concentrations do not change significantly, such as muscle protein, they still are important in energy metabolism during starvation.

Migration of the Silver eel is thought to be associated with a non-feeding state even though no experimental evidence exists since no eel has been caught during the sea phase of migration or at the spawning area; thus, the exact metabolic effects of migration are still unknown (Larsson and Fange, 1969; Lewander et al., 1974). Some data concerning the differences in metabolite concentrations of the immature yellow and silver European eel prior to its migration have been reported (Larsson and Fange, 1969; Johansson et al., 1974;

Lewander et al., 1974). Silver eels have lower quantities of liver glycogen but red and white muscle glycogen are similar at both stages. Plasma and liver proteins are higher in the silver eel while muscle proteins are lower. Silver eels were caught at the beginning of their migration and it is not surprising to find that fat content of all tissues is higher in the silver than yellow eel.

Like many fish, large quantities of fat accumulated in eel body tissues during growth are significantly depleted during food deprivation (Larsson and Lewander, 1973). It has also been proposed that gluconeogenesis from amino acids increases in liver during starvation to maintain glycogen stores and blood glucose (Dave et al., 1975). Hayashi and Ooshiro (1975) have shown that gluconeogenic rates from lactate are similar in perfused livers of the Japanese eel, A. japonica, compared to that reported for rat liver. Although the absolute need for glucose as an energy source in nerve tissues and red blood cells has not been demonstrated in any fish, no glucose was found in blood samples obtained from the bulbus arteriosus of hepatectomized Japanese eels (Inui and Yokote, 1977).

A tissue which may require large amounts of glucose, and is drained by the circulation prior to the heart, is the white muscle. Its metabolism is principally anaerobic with glucose as the main energy source since it has a poor vascularity, low stored glycogen and few mitochondria per fiber. Interestingly, white muscle constitutes the predominant muscle type with red muscle essentially absent, especially in the young immature Yellow eel (Hulbert and Moon, 1978). Even though fats are probably an important energy source in Anguilla spp., fat oxidation does not occur in white muscle, so if this muscle is used

for swimming activity glucose must be present and as shown by the results of Inui and Yokote (1977) liver is important in eel glucose homeostasis.

Therefore, the American eel represents an ideal system for biochemical studies since it is adapted to extensive periods of starvation and there is a need for glucose for white muscle locomotory activity, so an increase in gluconeogenesis during starvation would seem reasonable.

The purpose of this study was to establish the importance and the role of gluconeogenesis during fed and food deprived conditions in immature American eels using an eel hepatocyte preparation. The hepatocyte technique has been extensively used in the study of liver metabolism in mammals (Krebs et al., 1973). Even though a similar technique has been used to study glycogenolysis in goldfish (Birnbaum et al., 1976), the biochemical and physiological conditions of these hepatocytes have not been extensively studied. Therefore, the preliminary section (Chapter 3) of this study deals with some biochemical and physiological characteristics of the eel hepatocyte system and establishes the pathway and preferred substrates for eel liver gluconeogenesis. Finally (Chapter 4), gluconeogenic rates are established using hepatocytes prepared from starved eels up to 10 months using physiologically significant substrate concentrations. These results are discussed in light of the process of gluconeogenesis in fish and its importance during starvation in the American eel.

II

METHODS AND MATERIALS

METHODS AND MATERIALS

THE ANIMAL

Immature American eels, Anguilla rostrata, were collected from the Ottawa River at Ottawa, Ontario and from the eel ladder associated with the Cornwall hydro electric generating dam on the St. Lawrence River at Cornwall, Ontario. Fish were kept in tanks of circulating dechlorinated Ottawa tap water. Eels from the Ottawa River weighed between 300-600 gm and were kept at $15 \pm 1^{\circ}\text{C}$ without food until used, but no longer than two months. Eels from Cornwall weighed between 100-200 gm and were divided into fed and food deprived groups at either $15 \pm 1^{\circ}\text{C}$ or $5 \pm 1^{\circ}\text{C}$. Eels did not respond to feeding at 5°C , but the 15°C group was fed beef liver every day throughout the experimental period. It should be remembered that this diet is not like their natural diet.

HEPATOCYTE PREPARATION

Fish were anesthetized with MS 222 (ethyl-M-aminobenzoate methane sulfonic acid) for 5 min. An abdominal incision was made to expose the liver, and the hepatic portal vein was cannulated with PE 205 surgical tubing. The liver was removed and immediately perfused at 5 ml/min with an O_2 -saturated modified freshwater teleost ringer solution (Young, 1933) initially Ca^{++} -free (pH 7.4) to remove red blood cells. This initial perfusion was either continued a further 45 min with the same perfusion fluid to which was added 0.1%(w/v) collagenase and 0.1%(w/v) hyaluronidase (Berry and Friend, 1969), or it was followed by a 30 min perfusion with the ringer solution to which was added 1 mM Ca^{++} (Seglen, 1972), 0.1%(w/v) collagenase and 0.1%(w/v) hyaluronidase. Glucose was added to a final concentration of 5 mM in all perfusion media to pre-

vent excessive glycogen breakdown during cell preparation. At the end of perfusion, the cells were dispersed, centrifuged (Sorval RC-2B refrigerated centrifuge, 4 °C) at 50 g for 5 min and washed with about 15 ml fresh teleost ringer at pH 7.4. The yield of hepatocytes was determined by cell counting using a hemacytometer. Viable cells were those that excluded trypan blue (see explanation in Seglen, 1972).

CELL INCUBATION

Following cell washing, 30-50 mg wet weight of cells (approximately 10^6 cells) were suspended in 2 ml of a modified freshwater teleost ringer in 25 ml Erlenmeyer flasks. Phosphate buffer replaced the normal bicarbonate buffer to prevent saturation of the trapping fluid. Flasks were placed in a shaking water bath (Eberbach Model 6250) and gassed with 100% O₂ for 10 min. When radioactive substrates were added, hyamine hydroxide (0.2 ml, NEN Canada) was placed in a small container attached to the rubber stopper to trap radioactive CO₂.

Viability and biochemical and physiological properties of the prepared hepatocytes were studied by incubating the cells in one of four media: 1) the standard Ca⁺⁺-containing phosphate buffer, called the control medium; 2) Ca⁺⁺-free phosphate buffer; 3) 0.2%(w/v) albumin containing phosphate buffer; or 4) 1.5%(w/v) gelatine containing phosphate buffer. Eel hepatocyte glucose metabolism was studied by adding 1 µCi [U-¹⁴C]-glucose (NEN specific activity (SA), 213 mCi/mmol) or 1 µCi [6-¹⁴C]-glucose (NEN SA, 52.8 mCi/mmol) with or without the addition of bovine insulin to the flasks. Specific conditions are mentioned in the Table and Figure legends.

For the determinations of gluconeogenic rates, all livers of St. Lawrence River eels were perfused 30 min with the 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4, and the hepatocytes were incubated 1 hr in the standard Ca^{++} -containing phosphate buffer, pH 7.4, at the same temperature to which the fish were acclimated. Rates of gluconeogenesis were estimated from measuring radioactivity incorporated into glucose from 1 μCi [$\text{U}-^{14}\text{C}$]-lactate (NEN SA, 138.6 mCi/mole), 1 μCi [$\text{U}-^{14}\text{C}$]-alanine (NEN SA, 168 mCi/mole), 1 μCi [$\text{U}-^{14}\text{C}$]-aspartate (NEN SA, 192 mCi/mole) or 1 μCi [$\text{U}-^{14}\text{C}$]-glycerol (NEN SA, 8.75 mCi/mole) plus the appropriate cold substrate to the concentration listed in the Table and Figure legends. The effects of glucagon and food deprivation on glucose production at 5°C and 15°C were studied with the first three substrates while the effect of cortisol was studied from only 1 μCi [$\text{U}-^{14}\text{C}$]-lactate.

METABOLITE DETERMINATIONS

A) Analytical measurements of whole tissues

Metabolite concentrations were determined in plasma, liver and epaxial white muscle obtained from the area around the anus at each month of food deprivation, and from a piece of liver cut and deep frozen in liquid nitrogen just prior to perfusion. For the starvation studies, fish were anesthetized in MS 222 for 2 min and a blood sample was obtained from the caudal vein. Blood cells were removed by centrifugation and the plasma, together with the liver and white muscle, were deep frozen in liquid nitrogen and stored at -70°C until analysed. For glycogen determinations, a piece of tissue was hydrolyzed with 30% (w/v)

KOH, glycogen was extracted by alcoholic precipitation and hydrolyzed to glucose with 0.4 N sulfuric acid according to Good et al. (1933). The glucose obtained from the acidic glycogen hydrolysate was determined by the glucose oxidase-peroxidase method (Sigma bulletin #510) to which 2.5 ml 7.8 N sulfuric acid (reagent grade) was added to 2.0 ml of the incubate after chromagen formation. The resulting pink color was measured at 540 nm and was stable for a least 12 hrs. Proteins were measured with the Folin reagent with bovine serum albumin as a standard according to Lowry et al. (1951).

For other determinations, the plasma was deproteinized by adding sulfosalicylic acid crystals to a final concentration of 6%(w/v). Five hundred mg of liver or skeletal muscle were homogenized in 2.5 ml 6% (w/v) perchloric acid (or 6% sulfosalicylic acid in the study of starvation) with a Brinkmann Polytron PCU-2 homogenizer and the protein precipitate was removed by centrifugation in a IEC clinical centrifuge at full speed for 20 min. Glucose was determined by the glucose oxidase-peroxidase method described above. Amino acids were determined by the ninhydrin test according to Troll and Cannan (1953) using leucine as a standard. Since other substances also react with ninhydrin, results are expressed as ninhydrin positive substances. Lactate was determined enzymatically according to Hohorst (1963). The enzymatic determination of ATP was according to Lamprecht and Trautschold (1963) and of ADP/AMP according to Adam (1963). Sodium and potassium were measured by atomic emission (Jarrell Ash Model 82-270). Calcium was measured fluorimetrically with 3,6-dihydroxy-2,4-bis-[N,N'-(carboxymethyl)-aminomethyl] fluoran as the fluorescent substance. The increase in fluorescence upon addition of Ca^{++} was determined with a Turner Model 110 fluoro-

meter according to the Turner manual (1968).

B) Analytical measurements of hepatocyte metabolites

Wet weight of hepatocytes was estimated with the following procedure. Percent water in the intact liver was estimated by overnight drying at 100°C of a piece of liver cut just after cannulation (results obtained by this method did not differ significantly from those obtained by freeze-drying). The dry weight of hepatocytes was estimated by overnight drying (at 100°C) after centrifugation and discarding of the isolation fluid. Wet weight was calculated from dry weight using the percentage of water in the intact liver assuming that the water content remained constant during the hepatocyte preparation.

The O_2 uptake by the isolated hepatocytes was measured with a Gilson respirometer in 2.0 ml phosphate buffer using 0.2 ml 15% KOH as a carbon dioxide trap. Incubation medium glucose was determined by the glucose oxidase-peroxidase method as described above. For ion determinations, cells were washed with 2.0 ml 0.25 M sucrose, centrifuged and resuspended in 1.0 ml 6% perchloric acid. After vigorous shaking, the protein precipitate was removed by centrifugation in a IEC clinical centrifuge at full speed for 20 min. Sodium, potassium and calcium were measured from the supernatant as described above.

C) Radioactive measurements

When radioactive substrates were used, at the end of the incubation period the CO_2 trap was placed directly into scintillation fluid (see below) and the cells were centrifuged at 0°C for 3 min at 100 g. Incubation medium glucose was separated from other organic compounds by alusil thin layer chromatography using a butanol:acetic acid:water (6:3:1, v/v/v) solvent system according to Stahl and Kaltenbach (1965). Excess

salts, known to inhibit migration on the chromatograms, were removed with a strong cation exchanger which also removed positively charged amino acids. Lactate was found to migrate poorly in this solvent system and did not contaminate the glucose spot as determined by radiographic scanning. The separation of glucose from glycerol was achieved by alusil thin layer chromatography using a chloroform:acetone:5N ammonia (1:8:1, v/v/v) solvent system. The glucose and glycerol spots were separated by at least 3.5 cm.

The cell pellet remaining after centrifugation was treated with 2.0 ml 20% perchloric acid, 0.1 ml 1% sodium sulfate and 4.0 ml 99% ethanol containing 0.1% lithium chloride (for glycogen precipitation). After vigorous mixing, the solution was centrifuged at 39000 g for 20 min in a Sorval RC-2B at 0° C. The supernatant was decanted and subjected to gentle heating to evaporate the alcohol and an equal volume of chloroform-methanol (2:1, v/v) was added to the remaining fluid for lipid extraction. After centrifugation (IEC clinical centrifuge at full speed for 20 min), an aliquot from the chloroform layer was prepared for liquid scintillation counting. This fraction was called the lipid soluble fraction.

The remaining highspeed pellet was resuspended in 2.0 ml chloroform-methanol (2:1, v/v) and recentrifuged at 10000 g for 20 min to extract precipitated lipids. The pellet was further washed with 1.0 ml chloroform-methanol which was then added to the initial extract. An aliquot of this combined extract was prepared for radioactive counting. The radioactive counts estimated from the chloroform-methanol extracts of the pellet and supernatant of the first highspeed centrifugation were summated to give substrate incorporation into total lipid.

Proteins in the chloroform-methanol pellet were hydrolyzed with 1.5 ml 30% KOH in boiling water for 30-45 min. After cooling, 0.1 ml 1.0% sodium sulfate and 99% ethanol containing 0.1% lithium chloride were added to a final ethanol concentration of 66%. After standing overnight, glycogen was precipitated by centrifugation (IEC clinical centrifuge at full speed for 20 min). The resulting pellet was washed with 2.0 ml 65% ethanol. Glycogen was hydrolyzed with 0.4 ml 1.0 N sulfuric acid (reagent grade) in boiling water for 3 hrs. An aliquot was counted for radioactive substrate incorporation into glycogen and the glucose resulting from hydrolysis was determined with the glucose oxidase-peroxidase method as described above. Recoveries of glycogen and lipid with this method were found to be 95-99%.

A modification of the above standard extraction procedure was used to establish into which lipid fraction radioactive substrates were incorporated. At the end of the incubation period, cells were sedimented as described above, immediately resuspended and homogenized in 3 ml chloroform-methanol (2:1 v/v). After centrifugation (Sorval RC-2B centrifuge, 10 min at 3000 g), the pellet was resuspended in 1.0 ml chloroform-methanol (2:1 v/v) and recentrifuged. Supernatants from both centrifugation steps were combined and washed of their non-lipid radioactive substances with 2.0 ml of distilled water. Aliquots of the chloroform-methanol fraction were counted for radioactivity (this procedure, after value corrections, gave results which did not differ significantly from the initial procedure described above). The remaining volume of chloroform-methanol was evaporated by gentle heating. The lipids were saponified for 3 hr in an alcoholic-hydroxide solution and glycerol was extracted from the fatty acids and non-saponifiable lipids with

petroleum ether according to Entenman (1959). An aliquot of each fraction was prepared for radioactive counting.

RADIOACTIVE COUNTING

All radioactive counting was done with a Beckman L 233 scintillation counter. Quench corrections were estimated using quench curves made from quenched samples including all conditions described above and from Beckman quenched samples. Aquasol-2 (NEN, Canada) was used as a scintillation fluid. All activities (dpm) were converted to the corresponding mole quantities using the specific activity of the substrate in the incubation flask.

SAMPLE VARIABILITY AND STATISTICS

All results are expressed as the mean $\pm$ SEM as determined from duplicate experiments. The variability of the different analytical measurements was established by dividing the hepatocytes obtained from a single fish into six independent incubations with 1 μ Ci [U- 14 C]-lactate. Two fish from the St. Lawrence River group were tested in this manner. The SEM of the enzymic determinations of glucose and glycogen represented less than 5% of the mean of the six samples, while for the radioactive measurements of CO₂, lipid, glycogen and glucose, the SEM represented less than 10% of the mean (see Appendix 1). A paired t-test was used for statistical differences at the P < 0.05 level.

MATERIALS

All radioactive substrates were purchased from NEN Canada, Ltd. All chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri.

Other materials, including solvents, were purchased from local distributors and were of the highest possible purity.

III

GLUCONEOGENESIS IN THE EEL
HEPATOCYTE

INTRODUCTION

Four tissue preparations have been employed for the in vitro study of liver metabolism: liver homogenates, liver slices, liver perfusions and isolated hepatocytes (Walter, 1973; Shimassek et al., 1973; Story et al., 1976). The last two methods have numerous advantages over the first two and have been the preparations of choice over the past few years. In tissue homogenates, cellular integrity and ultra-structure are lost. Metabolic pathways depend upon a specific cellular architecture since many enzymes are either associated with membranes, such as the sarcoplasmic reticulum, or compartmentalized within organelles, such as mitochondria (Shimassek et al., 1973). Thus, in a homogenate system, metabolic controls are disturbed because enzymes can diffuse freely without regard to any specific spatial arrangements of pathways. Moreover, addition of substrates into a homogenate system may effect enzymes which previously were not exposed to them due to membrane barriers. It has also been shown that a severe loss of adenylates (ATP, ADP and AMP) and glycogen occurs in such preparations (Shimassek et al., 1973). Finally, hormones that interact with cell membrane receptors cannot be studied with the homogenate system. This preparation therefore is non-physiological and is no longer used for in vitro studies.

All other in vitro preparations avoid these problems since cellular integrity is maintained, substrates are added outside the cell and receptors for hormones remain intact. Tissue slices, however, present a problem of diffusion. Cells in the middle of a slice are not exposed directly to the medium and the availability of oxygen and

substrates is reduced as a function of distance from the medium. Thus, rates of uptake and/or synthesis can be underestimated (Walter, 1973). The diffusion problem is avoided with the perfusion or isolated hepatocyte systems since all cells are bathed either through the circulatory system or directly by the medium, and therefore are independent of one another in obtaining the required materials from the medium.

There have been three limitations ascribed to the hepatocyte preparation which are avoided by the liver perfusion. The first is a sodium gain and a potassium loss during the isolation of the hepatocytes (Berry and Friend, 1969; Krebs et al., 1973). It has been shown, however, that the addition of 1 or 4 mM Ca^{++} to the isolation perfusate yields hepatocytes which have normal Na^+ and K^+ contents and a higher O_2 uptake than cells prepared without added Ca^{++} (Seglen, 1972).

A second problem could be the turnover of substrates since hepatocytes remain suspended in the same medium for the entire incubation period. When a substrate, such as lactate, is taken up by a hepatocyte it could simultaneously be produced and released into the medium as a product of normal cellular metabolism. Similarly, a product, such as glucose, released into the medium could be reaccumulated by a hepatocyte. The net production of a particular substance would depend upon the difference between these two opposing fluxes (i.e., uptake and release). With the perfusion technique, the perfusate remains in contact with a cell only a short time, thus turnover rates would decrease and rates of metabolite production could be determined more precisely. It has been demonstrated, however, that rates of O_2 uptake, gluconeogenesis, ureogenesis, ketogenesis, adenylate content, permeability of the outer cell membrane and response to glucagon and

insulin are similar in both preparations of the rat liver (Johnson et al., 1972; Krebs et al., 1973). Story et al. (1976) have also demonstrated similar gluconeogenic rates in starved rat hepatocytes and starved perfused rat livers; however, the rate of gluconeogenesis from lactate is 2.2 times higher in fed rat hepatocytes than in fed perfused rat livers. These investigators have suggested that metabolic rates, and possibly controls, may be different in perfused livers and isolated hepatocytes when using fed rats.

The third limitation is that substrate concentrations vary. Furthermore, they cannot be altered during hepatocyte incubation. With the perfusion technique, substrate concentrations can be maintained throughout the entire experimental period, or specific modifications, such as substrate concentrations or hormone additions, can be made at specific times, and changes in metabolism following these modifications could be measured from the perfusate. These experiments are not easily achieved with incubated hepatocytes. A new technique, known as perfusion, has been recently developed. This technique consists of adding isolated cells to a column plugged at both ends with filters through which a buffer containing substrates or hormones is passed. Thus, as with perfusion, perfusion permits modifications in substrate and hormone concentrations during the experiment. When these techniques are compared, perfusion or incubated hepatocytes yield similar rates of gluconeogenesis and O_2 uptake (van der Meer et al., 1975). Glycogenolytic rates are also similar, but hormonal effects are different:

- 1) both glucagon and epinephrine stimulate glucose release from glycogen, but their effects last 10 min with the perfusion technique compared to 1 hr with the incubated hepatocytes; and 2) glucagon did

not stimulate gluconeogenesis from lactate in the perfusion system (Walter et al., 1975). Therefore, this perfusion technique requires further development before any results can be compared to other in vitro techniques.

Gluconeogenesis has been extensively studied in the rat liver using both the perfusion and the hepatocyte preparations. The best substrates, defined by estimating gluconeogenic rates in fed and starved rat livers, are lactate, glycerol, alanine, proline and serine in that order, (Ross et al., 1967a,b; Exton et al., 1970; Johnson et al., 1972; Garrison and Haynes, 1973). Aspartate and glutamate, which are deaminated to oxaloacetate (OXA) and α -ketoglutarate (α KG), are poor substrates. The hormones, epinephrine, glucagon and cortisol, increase glucose release by the rat hepatocyte and perfused liver preparations. Glucagon and epinephrine, via the second messenger cyclic AMP, increase glycogenolysis and decrease glycogenesis and glycolysis by a phosphorylation process of the key enzymes glycogen phosphorylase and glycogen synthetase (Hers et al., 1973; Birnbaum and Fain, 1977; Massague and Guinovart, 1977) and pyruvate kinase (Van Berkel et al., 1976; Clark, 1976; Riou et al., 1976; Rognstad, 1976). Gluconeogenesis from lactate, alanine and serine is increased in the liver by glucagon addition, but the exact mechanism for this increase remains uncertain (Ross et al., 1967a,b; Mallette et al., 1969; Garrison and Haynes, 1973). Cortisol, unlike glucagon and epinephrine, penetrates the cell membrane and together with a cytosolic receptor activates protein synthesis (Baulieu, 1975) which ultimately increases both glycogenesis (Kendysh and Moroz, 1972) and gluconeogenesis (Krone et al., 1976) mainly from amino acids (Waterlow, 1968; Kendysh and Moroz, 1972).

Similar studies are only just now being done using fish as experimental animals.

Gluconeogenesis from alanine, in vivo, has been shown in the rainbow trout Salmo gairdneri. Rates are lower in trout fed a high carbohydrate diet than in trout fed a high protein diet or food deprived for one month (Cowey et al., 1977a). Gluconeogenesis from lactate has also been demonstrated in lamprey and eel liver slices (Phillips and Hird, 1977) and in perfused eel liver (Hayashi and Ooshiro, 1975). In vivo, insulin inhibits gluconeogenesis from alanine and has a pronounced hypoglycemic effect in the rainbow trout (Cowey et al., 1977a,b) and European eel, Anguilla anguilla (Lewander et al., 1976). Insulin also inhibits glycogenolysis and gluconeogenesis in the perfused eel (A. japonica) liver while cAMP and epinephrine have a reverse effect (Hayashi and Ooshiro, 1975). Birnbaum et al. (1976) have shown that catecholamine-stimulated glycogenolysis in goldfish (Carassius auratus hepatocytes is probably a β -adrenergic effect. Both glucagon and epinephrine have a hyperglycemic effect in vivo, but only the latter has a lipolytic effect in the European eel (Larsson and Lewander, 1972; Larsson, 1973), while cortisol stimulates glycogenesis in liver and muscle of the American eel, A. rostrata (Butler, 1968). Therefore, the control over fish gluconeogenesis may be similar to that reported in mammals.

In this chapter, gluconeogenic rates and the control of these rates by glucagon and cortisol are studied in hepatocytes prepared from fed American eels, A. rostrata. The isolated hepatocyte preparation was chosen over a liver perfusion system to study eel gluconeogenesis for two reasons. Firstly, with liver perfusion, each liver

can be perfused with only one substrate at a time; therefore, as the number of substrates and experimental conditions increases so does the number of livers required. An isolated hepatocyte suspension can be divided into many samples each of which receives a different substrate or is exposed to a different experimental condition.

Secondly, a population of eels may not necessarily be homogenous and large variations in gluconeogenic rates might be expected. Statistical differences between substrates could be difficult to discern under these conditions. With the hepatocyte technique, gluconeogenic rates from different substrates can be estimated from the same liver and even if the variability of a population is large, the relative difference between substrates will be statistically meaningful. Thus, the choice of the hepatocyte technique was determined more by the availability of tissue than the precision of the experimental method.

The fish hepatocyte has not been characterized extensively, even though goldfish hepatocytes have been used to study glycogenolysis. Birnbaum et al. (1976) have reported that 90% of the isolated goldfish hepatocytes exclude trypan blue and that adenylate contents are maintained over 2 hrs of incubation. Therefore, the eel hepatocyte preparation is examined for its viability and biochemical and physiological properties followed by a study of the preferred gluconeogenic substrates and the pathway by which these substrates are converted to glucose or other cellular intermediates. The results indicate that the eel hepatocyte system behaves in a manner qualitatively similar to the rat system, although major quantitative differences were noted which probably arise from different dietary compositions.

RESULTS

I. HEPATOCTYTE VIABILITY

A) Effects of perfusion

Perfusion of the Ottawa River eel liver with a Ca^{++} -free medium resulted in a hepatocyte preparation of which about 88.5% excluded trypan blue (Table 1). Addition of 1 mM Ca^{++} to the perfusate increased this percentage to 93% in a single experiment. Similarly, 91.8% of the hepatocytes prepared from the St. Lawrence River eel livers perfused with a 1 mM Ca^{++} -containing bicarbonate buffer, excluded trypan blue (Table 2). Red blood cell contamination increased when calcium was added to the perfusate (Table 1). Cell numbers average 2×10^6 cells per 100 mg for all perfusion conditions.

Hepatocyte glycogen declined by approximately 35% following perfusion with or without Ca^{++} (Tables 1 and 2). No significant differences were observed in the protein content of the hepatocytes, but the ionic composition changed markedly (Table 1). Perfusion of the liver in the absence of Ca^{++} resulted in a large gain in Na^+ and a loss in both K^+ and Ca^{++} from the hepatocytes. With Ca^{++} addition, a further increase of Na^+ occurred while no significant changes were noted in K^+ and Ca^{++} levels when compared to the whole liver (Table 1). Respiration rates of the isolated hepatocytes were linear for at least 2 hrs and the presence of 1.0 mM Ca^{++} in the perfusate increased these rates by about 50% in the absence of substrates (Table 1).

B) Effects of glucose, albumin and gelatine

Since a saline teleost ringer-phosphate buffer is physiological but not the normal circulating fluid, an attempt was made to optimize

Table 1. Effects of the presence of Ca^{++} during eel liver perfusion on the properties of the hepatocytes. Livers from Ottawa River eels were perfused either 45 min with a Ca^{++} -free bicarbonate medium, pH 7.4, or 30 min with a 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4. Liver concentrations were estimated from a piece of liver cut and frozen in liquid nitrogen just before perfusion. Results are expressed as MEAN $\pm$ SEM and numbers in parentheses represent numbers of fish assayed.

¹Percentage of hepatocytes that excluded trypan blue.

²Percentage of red blood cells to the total number of cells present.

³Oxygen uptake in the absence of substrate.

*Significantly different from liver concentration; t-test
P<0.05

PARAMETERS	PERFUSATE (1mM Ca ⁺⁺)	LIVER CONCENTRATION	HEPATOCYTES
Membrane integrity ¹	-		88.5 ± 1.8 % (6)
	+		93.3 % (1)
RBC contaminations ²	-		4.8 ± 0.3 % (6)
	+		12.0 % (1)
Yield of cells (no. cells x 10 ⁶ /mg)	-		3.07 ± 1.50 (6)
	+		1.12 ± 0.24
Glycogen (mg/100 mg)		3.78 ± 0.47 (10)	2.33 ± 0.33 (10) *
	+	6.43 ± 0.52 (2)	4.47 ± 0.23 (2) *
Protein (mg/100 mg)	-	17.70 ± 0.73 (5)	19.07 ± 1.62 (2)
Na ⁺ (μeq/100 mg)	-	0.52 ± 0.02 (4)	3.39 ± 1.05 (4) *
	+	0.57 ± 0.12 (2)	4.87 ± 0.60 (2) *
K ⁺ (μeq/100 mg)	-	3.97 ± 0.14 (4)	2.01 ± 0.21 (4) *
	+	4.12 ± 0.07 (2)	4.47 ± 0.89 (2)
Ca ⁺⁺ (μeq/100 mg)			*
	-	1.31 ± 0.16 (4)	0.64 ± 0.09 (4)
	+	0.70 ± 0.08 (2)	1.08 ± 0.33 (2)
O ₂ uptake ³ (μl O ₂ /hr/100 mg)	-		47.35 ± 6.58 (4)
	+		61.50 ± 10.3 (2)

Table 2. Characteristics of the hepatocytes prepared from St. Lawrence River eel livers perfused 30 min with a 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4. Results are expressed as the MEAN $\pm$ SEM and numbers in parentheses represent numbers of fish assayed.

^{1,2}As estimated in Table 1.

³Liver concentration of glycogen was 8.20 $\pm$ 0.88 mg/100 mg wet weight.

*Significantly different from liver concentration; t-test $P < 0.05$.

PARAMETERS	HEPATOCYTES
Membrane integrity ¹	91.8 ± 2.0 % (7)
RBC contaminations ²	11.3 ± 1.3 % (7)
Yields of cells (no. cells x 10 ⁶ /mg	2.38 ± 0.40 (7)
Glycogen ³ mg/100 mg)	5.26 ± 0.62 (8)*

the characteristics of the eel hepatocyte preparation by altering the components of this incubation medium. Therefore, 10 mM glucose, 0.2% albumin and 1.5% gelatine were added to the incubation medium of hepatocytes prepared in the presence or absence of Ca^{++} . All changes were compared to control experiments and to the initial values estimated from hepatocytes prior to incubation (Tables 3 and 4).

The percentage of hepatocytes which excluded trypan blue and the total number of cells per ml did not change significantly following 2 hrs of incubation in a normal Ca^{++} -phosphate buffer with or without 0.2% albumin (Table 3). Albumin slightly increased respiration rates of the hepatocytes but markedly enhanced the glucose stimulation of respiration. The hepatocytes released protein during incubation but this release was blocked by albumin (Table 3). No significant changes in ionic composition were observed after 2 hrs of incubation with the exception of a small gain of Ca^{++} when albumin was added to the incubate. Glucose addition generally maintained Na^+ and K^+ levels higher than in its absence (Table 3).

Removal of Ca^{++} from the incubation medium resulted in significant changes as would be predicted from Table 1. The percentage of hepatocytes which excluded trypan blue increased slightly but albumin reduced this value to below pre-incubation values (Table 3). The cell numbers per ml decreased significantly during incubation and this decrease was exaggerated by albumin. Respiration rates were greater than when Ca^{++} was present but the effects of 10 mM glucose and albumin were contradictory probably as a result of the small number of experiments attempted. Hepatocytes released large quantities of protein in the Ca^{++} -free medium, but albumin addition prevented this

Table 3. Effects of glucose and albumin on isolated eel hepatocyte characteristics. Livers from two Ottawa River eels were perfused 45 min with a Ca^{++} -free bicarbonate buffer, pH 7.4 and the isolated hepatocytes were incubated 2 hrs at $15 \pm 1^\circ \text{C}$ with or without Ca^{++} added to the teleost ringers. Results are from duplicate assays on each of two fish and are expressed as the MEAN $\pm$ SEM.

¹Added proteins were subtracted to obtain net gain or loss of proteins to the medium.

*Significantly different from values estimated in hepatocytes before incubation; t-test $P \leq 0.05$.

[§]Significantly different from values observed in hepatocytes after incubation in the standard Ca^{++} -phosphate buffer, pH 7.4; t-test $P < 0.05$.

PARAMETERS	PRE- INCUBATION	Ca ⁺⁺ Free Phosphate Buffer		Ca ⁺⁺ (1mM) Phosphate Buffer	
		None	0.2% Albumin	None	0.2% Albumin
<u>Membrane integrity</u>					
- % viability	84.7 ± 2.3	92.9 ± 2.5	76.5 ± 2.6*	86.3 ± 2.3	83.9 ± 2.0
- Total no. cells (cells x 10 ⁷ /ml)	1.52 ± 0.22	1.44 ± 0.16*§	1.05 ± 0.20*§	1.66 ± 0.56	1.74 ± 0.26
<u>O₂ uptake</u> (μl O ₂ /hr/100 mg)					
- Experiment 1: none		99.8		64.4	
10 mM glucose		64.4			
- Experiment 2: none		39.7	20.8	27.3	30.4
10 mM glucose		55.6		29.8	45.6
<u>Medium protein¹</u> (mg/100 mg)	0.32 ± 0.08	1.14 ± 0.30*	0.04 ± 0.01*§	0.64 ± 0.09	0.29 ± 0.33
<u>Ion compositions</u> (μeq/100 mg)					
Na ⁺ none	1.80 ± 0.33	2.85 ± 0.09*§	1.35 ± 0.02	1.43 ± 0.07	1.36 ± 0.15
10 mM glucose		3.29 ± 0.01*§		1.66 ± 0.10	1.70 ± 0.11
K ⁺ none	1.55 ± 0.30	1.69 ± 0.14	1.05 ± 0.03*§	1.69 ± 0.01	1.76 ± 0.07
10 mM glucose		1.87 ± 0.09		1.93 ± 0.06	1.97 ± 0.21
Ca ⁺⁺ none	0.50 ± 0.06	0.56 ± 0.02	0.30 ± 0.02*§	0.58 ± 0.03	0.68 ± 0.01*
10 mM glucose		0.50 ± 0.05		0.55 ± 0.04	0.66 ± 0.01

release (Table 3). In the absence of albumin, hepatocytes gained Na^+ , K^+ and Ca^{++} while albumin addition reduced all ion levels measured below the pre-incubation level. Glucose altered Na^+ and K^+ balance, but not Ca^{++} (Table 3).

Gelatine addition enhances rat hepatocyte dispersion (Katz et al., 1975) and therefore its effects were tested in the eel hepatocyte system (Table 4). As in Table 1, addition of 1 mM Ca^{++} to the perfusate increased respiration rates and glucose enhanced this effect. The glucose effect was further exaggerated by gelatine addition. In the absence of Ca^{++} in the perfusate, gelatine had no effect but glucose decreased respiration rates. Ca^{++} had no major effect on protein release by the hepatocytes. In general, levels of Na^+ , K^+ and Ca^{++} decreased to lower values during a 1 hr incubation independent of the perfusion system used but these effects were reversed back towards pre-incubation levels by both glucose and gelatine alone or when added together.

The major problem with gelatine is the formation of a gel when cells are incubated at 15°C. Thus, it was impossible to estimate the number of cells in the medium or the amount of protein in the medium.

C) Glucose metabolism in the eel hepatocyte

As a result of the above experiments, all livers were perfused with teleost ringers containing 1.0 mM Ca^{++} . [U-¹⁴C]-glucose metabolism at various concentrations of added cold glucose was studied in each of the above described three media. Glucose uptake by the isolated hepatocytes was measured by two methods: 1) estimating medium glucose at the end of the incubation period by the glucose oxidase-peroxidase method which gives net glucose uptake; and

Table 4. Effects of gelatine on hepatocytes isolated from the Ottawa River eels. Livers were perfused 45 min with a Ca^{++} -free bicarbonate buffer, pH 7.4, or 30 min with a 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4. Hepatocytes were incubated in the standard Ca^{++} -phosphate buffer, pH 7.4, for 1 hr with or without 10 mM glucose in duplicate at $15 \pm 1^\circ \text{C}$. Results are expressed in MEAN $\pm$ SEM of the duplicate samples.

*Significantly different from values observed in hepatocytes before incubation; t-test $P < 0.05$.

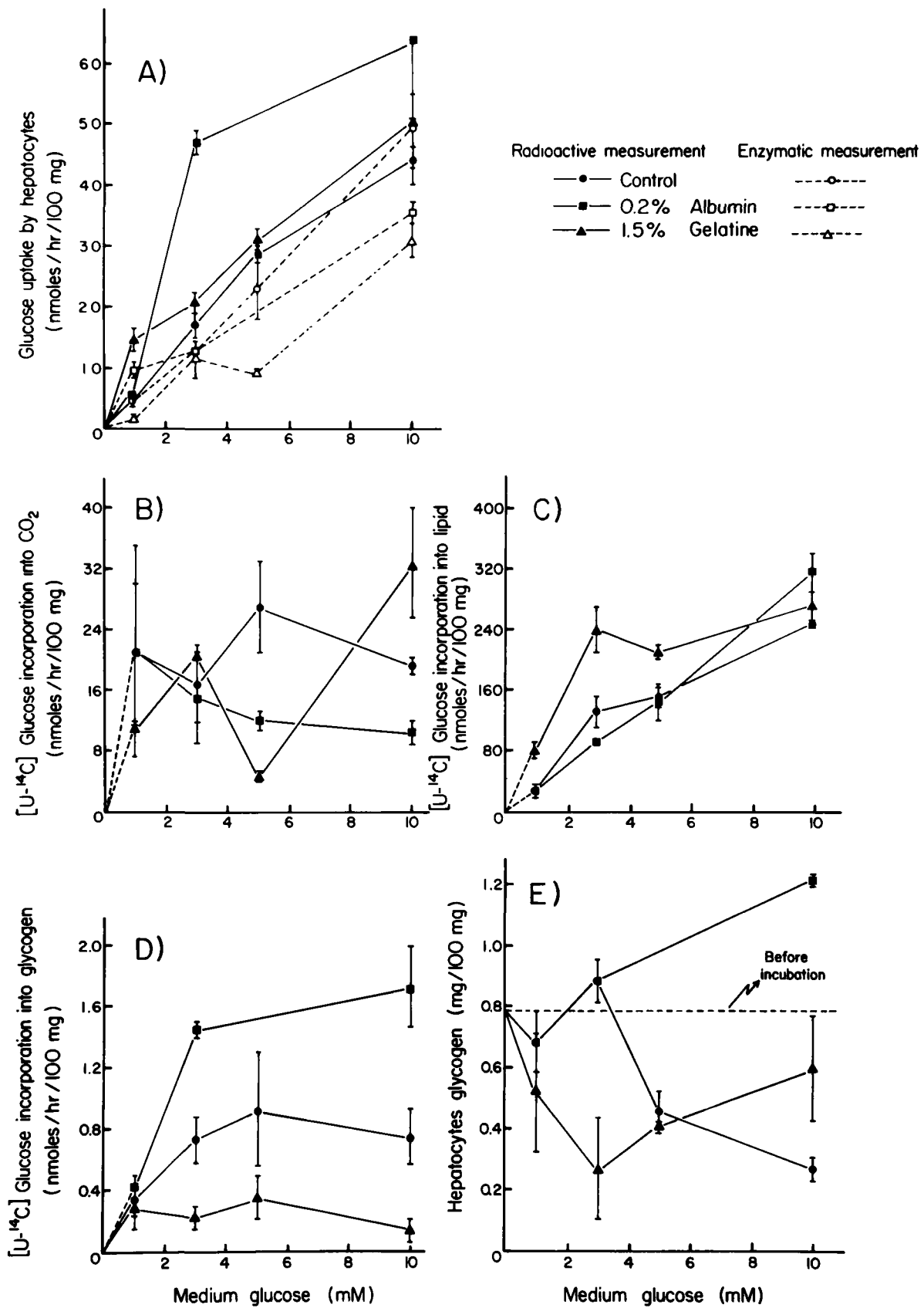
^sSignificantly different from values observed in hepatocytes prepared with a 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4, and incubated in the standard Ca^{++} -phosphate buffer, pH 7.4; t-test $P < 0.05$.

PARAMETERS	MEDIUM GLUCOSE (10 mM)	Ca ⁺⁺ Free perfusate			1 mM Ca ⁺⁺ containing perfusate		
		PRE- INCUBATION	NONE	1.5% GELATINE	PRE- INCUBATION	NONE	1.5% GELATINE
<u>O₂ uptake</u> (μ l O ₂ /hr/100 mg)	-		60.8 $\pm$ 5.9	65.7 $\pm$ 10.8		78.9 $\pm$ 4.3	83.2 $\pm$ 25.8
	+		49.1 $\pm$ 2.0§	43.1 $\pm$ 5.1§		91.8 $\pm$ 1.2	120.5 $\pm$ 10.2
<u>Medium protein</u> (mg/100 mg)	-		0.50 $\pm$ 0.01			0.46 $\pm$ 0.01	
	+		0.48 $\pm$ 0.05			0.17 $\pm$ 0.01	
<u>Ion compositions</u> (μ g/100 mg)							
Na ⁺	-	5.00 $\pm$ 1.27	1.77 $\pm$ 0.01	2.36 $\pm$ 0.56	4.87 $\pm$ 0.06	2.58 $\pm$ 0.20	4.88 $\pm$ 0.20§
	+		1.28 $\pm$ 0.21§	4.31 $\pm$ 0.10		3.44 $\pm$ 0.11	8.61 $\pm$ 0.20*§
K ⁺	-	2.33 $\pm$ 0.21	2.69 $\pm$ 0.12	1.76 $\pm$ 0.01§	4.47 $\pm$ 1.09	3.47 $\pm$ 0.15	4.30 $\pm$ 0.32
	+		2.06 $\pm$ 0.10§	2.19 $\pm$ 0.05§		4.30 $\pm$ 0.25	5.59 $\pm$ 0.24
Ca ⁺⁺	-	0.78 $\pm$ 0.09	0.36 $\pm$ 0.11*	0.88 $\pm$ 0.03	1.08 $\pm$ 0.32	0.62 $\pm$ 0.06	1.30 $\pm$ 0.09§
	+		0.42 $\pm$ 0.10	0.84 $\pm$ 0.05		0.64 $\pm$ 0.03	1.36 $\pm$ 0.23§

2) summing radioactivity incorporated from [U- ^{14}C]-glucose into hepatocyte solutes (i.e., CO_2 , lipid, glycogen and supernatant materials of the first highspeed centrifugation after lipid extraction; see Methods and Materials) which gives the apparent uptake of glucose (see Katz et al., 1975; Katz and Rognstad, 1976). Results of these two methods are presented in Fig. 1A. The two estimates in the control experiment did not differ significantly and glucose uptake increased linearly as medium glucose concentrations increased indicating that glucose release from the hepatocytes does not significantly contribute to medium glucose. In these experiments, about 25% of the added glucose (^{14}C + cold) was taken up by the hepatocytes at each medium glucose concentration after the 1 hr incubation. The addition of either albumin or gelatine inhibited net glucose uptake by the hepatocytes especially at high medium glucose concentrations, although the apparent glucose uptake was greater than controls (Fig. 1A). These results suggest that glucose release may be activated by these two proteins. In these experiments, the net glucose uptake by the hepatocytes was about 20% of the added glucose at low medium concentrations, but less than 15% at high medium glucose. The apparent glucose uptake, however, varied between 30 and 50% of added [U- ^{14}C]-glucose.

[U- ^{14}C]-glucose oxidation was independent of medium glucose concentrations from 1 to 10 mM in control experiments (Fig. 1 B). Addition of 0.2% albumin decreased [U- ^{14}C]-glucose oxidation while results with 1.5% gelatine were inconclusive. Incorporation into the lipid fraction increased linearly with glucose concentration in all three media. With 1.5% gelatine addition, values exceeded those of the control at 3 and 5 mM glucose (Fig. 1 C). The rate of incorpora-

Figure 1. Effects of albumin and gelatine on glucose metabolism in isolated eel hepatocytes. Livers from two Ottawa River eels were perfused with a 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4, and the isolated hepatocytes were incubated 1 hr in duplicate at $15 \pm 1^\circ \text{C}$ with 1 μCi [$\text{U}-^{14}\text{C}$]-glucose and cold glucose to give the indicated final medium glucose concentration in the standard Ca^{++} -phosphate buffer, pH 7.4. Samples were analysed for radioactivity incorporated into the various fractions as noted in Methods and Materials.



tion into glycogen was the lowest observed and appeared to saturate (Fig. 1 D). Albumin increased these rates by two-fold above control, while gelatine significantly inhibited rates. Fig. 1 E demonstrates that hepatocyte glycogen generally falls when cells are incubated in glucose concentrations in excess of 3 mM, and only albumin addition prevents this significant decline.

Since Ottawa River eels are more difficult to obtain than those from the St. Lawrence River, the latter eels were used for further studies. These eels are smaller but hepatocyte viability, cell yield and the glycogen loss during perfusion was similar to the hepatocytes from the Ottawa River eels (Table 2). The glucose metabolism of these hepatocytes was checked as in Fig. 1 for the Ottawa River eel hepatocytes (Table 5).

In the absence of substrates, the St. Lawrence River eel hepatocytes released 6.22 μ moles of glucose per hour per 100 mg wet weight. Addition of 3 mM glucose significantly decreased this output, but bovine insulin was needed to elicit a net glucose uptake (Table 5). Since $[6-^{14}\text{C}]$ -glucose was used in these experiments instead of $[\text{U}-^{14}\text{C}]$ -glucose, the apparent glucose uptake was determined by measuring the amount of radioactive glucose lost from the medium during incubation after purification of radioactive glucose on thin layer chromatography (see Methods and Materials). Interestingly, the apparent glucose uptake was less than 1% in these experiments, which is much lower than the percentage observed for the Ottawa River eel hepatocytes (15 to 30%; see above).

Regardless of medium additions, hepatocyte glycogen was always significantly depleted after a 1 hr incubation when compared to pre-

Table 5. Glucose metabolism in hepatocytes from St. Lawrence River eels.

Livers from four fed eels were perfused and hepatocytes incubated as described in Fig. 1, except that [14 C]-glucose was replaced by [14 C]-glucose. Results are expressed as MEAN $\pm$ SEM. Negative signs indicate that glucose was lost from the medium while positive signs indicate a net gain in medium glucose above that initially added.

¹Pre-incubation value of hepatocyte glycogen was 6.5 $\pm$ 0.92 mg/100 mg wet weight and was significantly different from all hepatocyte glycogen values observed after a 1 hr incubation; t-test P<0.05.

*Significantly different from values observed in hepatocytes incubated with 3 mM glucose; t-test P<0.05.

[§]Significantly different from values observed in hepatocytes incubated with no added substrate; t-test P<0.05.

PARAMETERS	ADDITION IN MEDIUM			
	NONE (CONTROL)	3 mM GLUCOSE	3 mM GLUCOSE 8.3 μ M INSULIN	10 mM GLUCOSE
<u>ENZYMATIC DETERMINATION</u>				
- MEDIUM GLUCOSE (μ moles/hr/100 mg)	+6.22 $\pm$ 0.72	+2.22 $\pm$ 0.66§	-0.06 $\pm$ 0.88*§	-9.99 $\pm$ 2.83*§
- GLYCOGEN ¹ (mg/100 mg)	4.55 $\pm$ 0.70	4.88 $\pm$ 0.50§	4.86 $\pm$ 0.51§	5.48 $\pm$ 0.76§
<u>¹⁴C₆ - glucose Incorporation</u> (nmoles/hr/100 mg)				
into - CO ₂		2.52 $\pm$ 0.24	3.21 $\pm$ 0.88	7.03 $\pm$ 1.83*
- lipid		60.50 $\pm$ 4.17	64.65 $\pm$ 8.03	180.03 $\pm$ 17.54*
- glycogen		8.34 $\pm$ 1.71	7.55 $\pm$ 1.62	16.79 $\pm$ 3.53*
- protein		0	0	0
- total		71.36	75.41	203.85

incubation hepatocyte glycogen (Table 5). Glycogen depletion, however, compared to control was prevented by glucose addition, but insulin did not enhance this level.

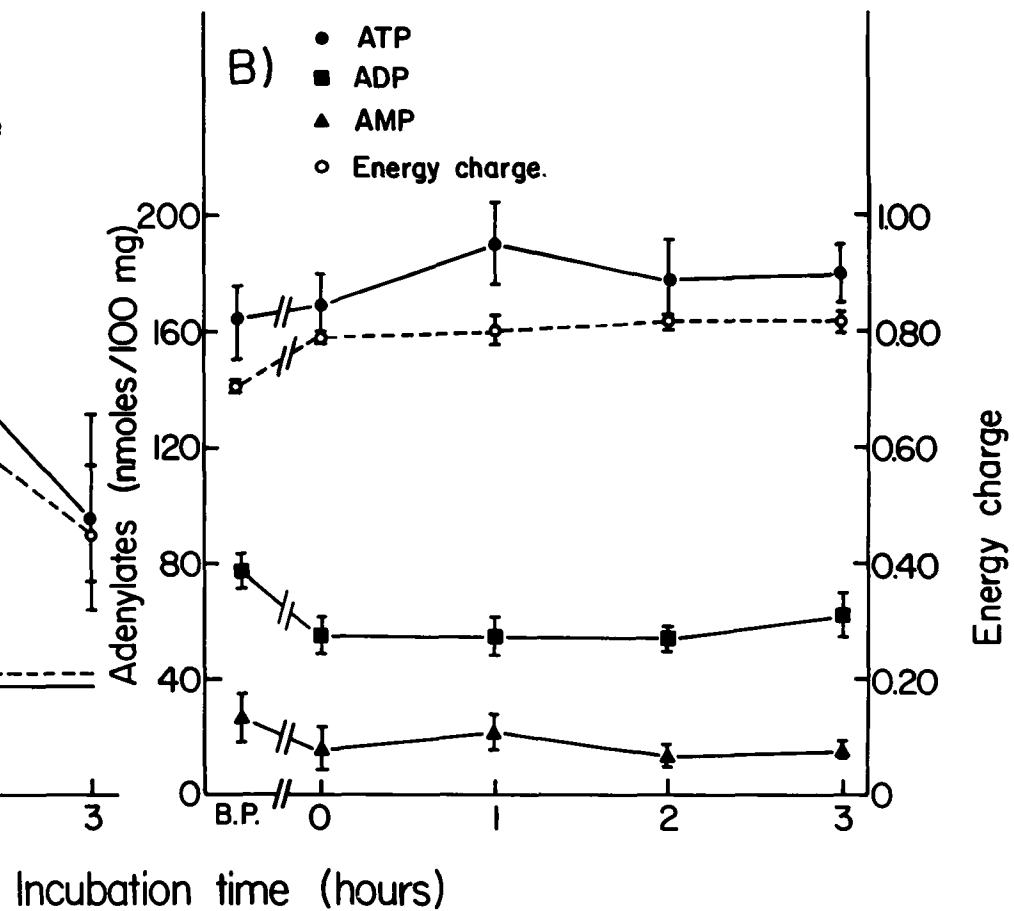
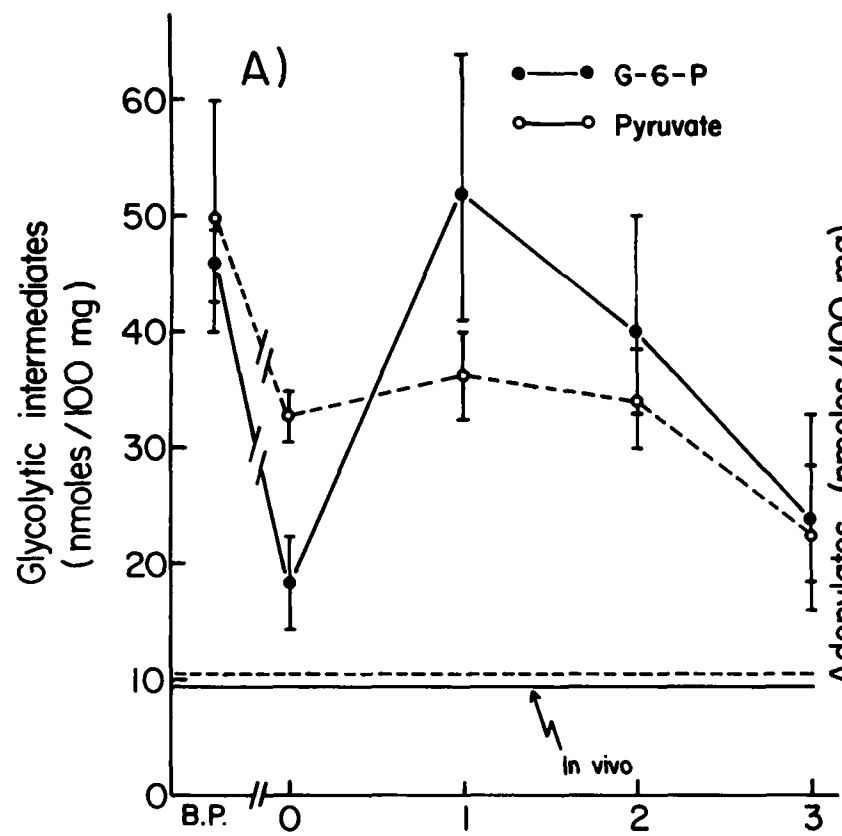
Radioactivity incorporated into CO_2 , lipids, and glycogen were much lower at all glucose concentrations than found for the Ottawa River eel hepatocytes (compare Table 5 with Fig. 1). Insulin did not significantly effect total incorporation. Only with insulin addition is there a significant correlation between total radioactivity and medium glucose uptake. Results with 10 mM glucose are difficult to interpret, but the enzymic determination of glucose should be rejected due to a large SEM value.

D) Energy charge of the hepatocytes

The maintenance of cellular metabolite concentrations and specifically the adenylates ATP, ADP and AMP, has been used to establish the biochemical integrity of hepatocyte preparations (Krebs et al., 1973; Shimassek et al., 1973). These concentrations, plus glucose -6- phosphate (G-6-P) and pyruvate, were estimated as a function of incubation time (Fig. 2). A 30 min perfusion with the standard Ca^{++} containing buffer reduced G-6-P and pyruvate concentrations significantly from those seen in a whole frozen liver piece removed before perfusion. During the incubation, G-6-P levels increased back towards the pre-perfusion level followed by a gradual decline (Fig. 2A). Pyruvate concentrations remained constant for 2 hrs but decreased slightly by 3 hrs of incubation (Fig. 2A). The hepatocytes maintained ATP, ADP and AMP concentrations and therefore the energy charge constant throughout the perfusion and incubation periods (Fig. 2 B).

These metabolite concentrations were also estimated in livers of

Figure 2. Metabolite levels in isolated eel hepatocytes. Livers from four fed St. Lawrence River eels were perfused and hepatocytes were incubated without substrate as described in Fig. 1. Before perfusion (BP) values were obtained from a piece of liver frozen immediately upon removal of the liver from the animal and in vivo means the normal liver concentrations of metabolites estimated from 4 eels killed by decapitation and immediately frozen. Vertical bars represents SEM. A) Glycolytic intermediates; B) Adenylate content.



four eels killed by decapitation and immediately frozen in liquid nitrogen. The values obtained represent the normal concentrations found in vivo in Fig. 2A. Between anesthesia and perfusion, the G-6-P and pyruvate concentrations increased and remained above normal throughout the incubation (Fig. 2 A); no significant change in adenylate concentrations was observed during the same time period (results not shown).

II. GLUCONEOGENESIS

A) Gluconeogenic rates in eel hepatocytes

The gluconeogenic pathway in the rat liver has been extensively studied (see Introduction). Liver glycogen after a 48 hr fast in the rat, is essentially zero and the only source of glucose released during liver perfusion or hepatocyte incubation must be from added gluconeogenic substrates. Eel liver glycogen, however, even after five months of starvation is still approximately 1.0 mg (10 μ moles glucose equivalent) per 100 mg of wet tissue weight. Thus, the estimation of gluconeogenesis in eel hepatocytes presents a unique problem.

In the absence of substrates, eel hepatocytes released approximately 5.0 μ moles glucose per hour per 100 mg wet hepatocyte weight into the medium (Table 6). Addition of 10 mM lactate, 10 mM alanine or 10 mM glycerol significantly reduced glucose release by 1.3-1.2- and 1.6-fold, respectively, while 10 mM aspartate had no effect (Table 6). Hepatocytes were also incubated with gluconeogenic inhibitors: 10 mM quinolinic acid which inhibits cytosolic PEP CK; 10 mM phenylpyruvate which inhibits PC; and 10 mM aminooxyacetate which inhibits cytosolic amino acid transaminases. These gluconeogenic

Table 6. Glucose release and glycogen depletion in isolated eel hepatocytes. Livers from fed St. Lawrence River eels were perfused and hepatocytes were incubated with the indicated substrates as described in Fig. 1. Results are expressed as the MEAN $\pm$ SEM and number in parentheses represent the number of fish assayed.

¹The pre-incubation value for hepatocyte glycogen was 4.87 ± 0.37 mg/100 mg wet weight and was significantly different from all hepatocyte glycogen values observed after a 1 hr incubation; t-test $P < 0.05$.

*Significantly different compared to no substrate addition; t-test $P < 0.05$.

§Significantly different compared to 10 mM substrate addition; t-test $P < 0.05$.

ADDED SUBSTRATES	GLUCOSE RELEASE (μ moles/100 mg)	HEPATOCYTE GLYCOGEN ¹ (mg/100 mg)
None	4.98 $\pm$ 0.62 (24)	3.68 $\pm$ 0.29 (24)
10 mM lactate	3.98 $\pm$ 0.73 (12) *	3.23 $\pm$ 0.29 (12) *
+ 10 mM quinolinic acid	4.58 $\pm$ 0.73 (8)	3.44 $\pm$ 0.50 (8)
+ 10 mM phenylpyruvate	4.08 $\pm$ 1.02 (8)	3.40 $\pm$ 0.38 (8)
10 mM alanine	4.30 $\pm$ 0.66 (16) *	2.99 $\pm$ 0.44 (16) *
+ 10 mM quinolinic acid	6.45 $\pm$ 1.86 (4)	2.64 $\pm$ 0.35 (4)
+ 10 mM phenylpyruvate	5.47 $\pm$ 1.55 (4)	2.63 $\pm$ 0.25 (4)
+ 10 mM aminooxyacetate	4.67 $\pm$ 1.00 (4)	2.92 $\pm$ 0.35 (4)
10 mM aspartate	5.07 $\pm$ 0.54 (14)	2.98 $\pm$ 0.35 (14) *
+ 10 mM quinolinic acid	10.05 $\pm$ 3.04 (2)	2.29 $\pm$ 0.44 (2)
+ 10 mM phenylpyruvate	5.73 $\pm$ 1.56 (2) §	2.15 $\pm$ 0.07 (2)
+ 10 mM aminooxyacetate	4.06 $\pm$ 0.72 (4) §	3.61 $\pm$ 0.38 (4)
10 mM glycerol	3.13 $\pm$ 0.44 (14) *	3.48 $\pm$ 0.35 (14)
+ 10 mM quinolinic acid	2.67 $\pm$ 0.11 (2)	3.70 $\pm$ 0.19 (2)
+ 10 mM phenylpyruvate	2.52 $\pm$ 0.01 (2)	3.49 $\pm$ 0.29 (2)

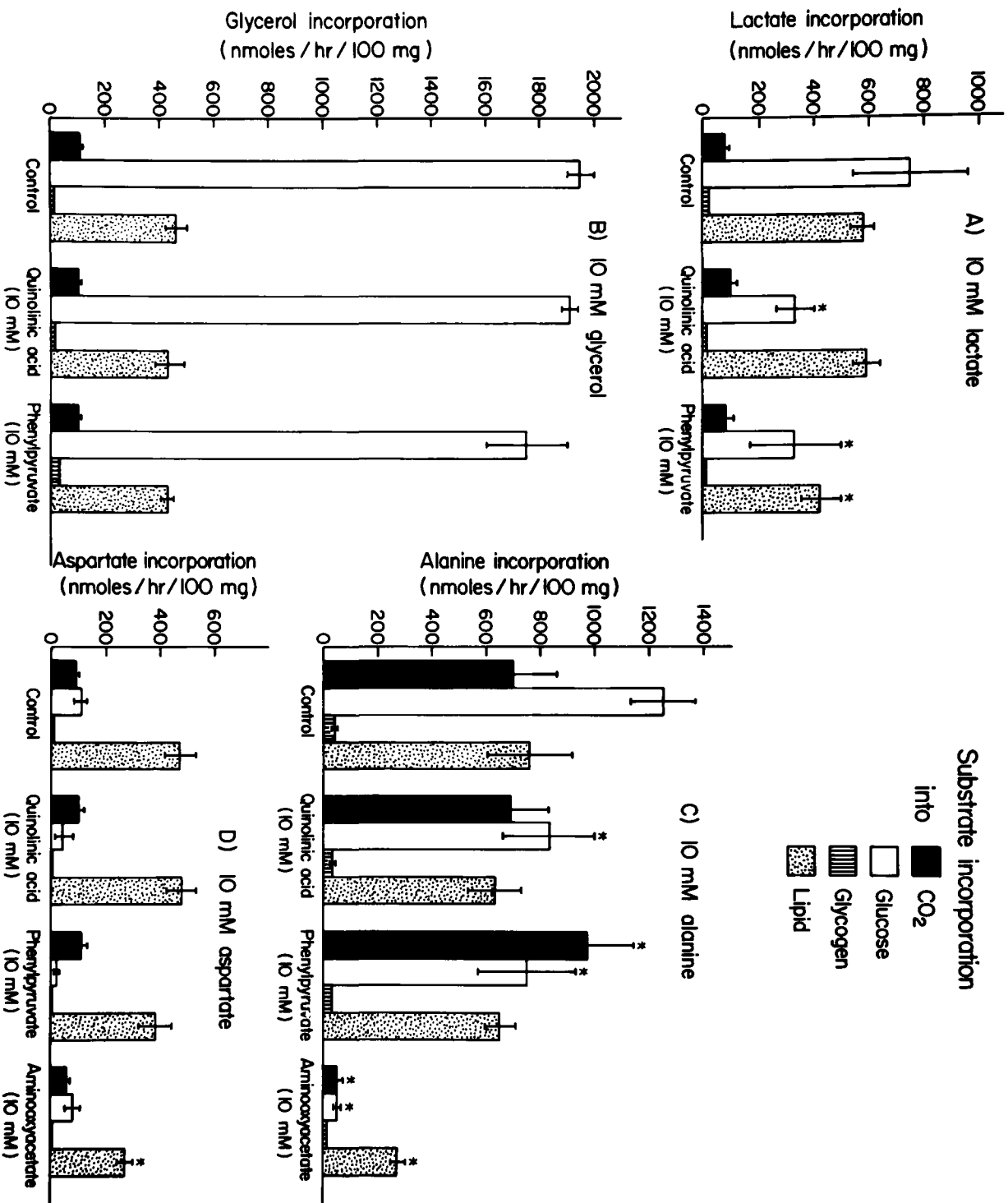
inhibitors partially removed lactate and alanine inhibition but enhanced glycerol inhibition of glucose release. Hepatocytes incubated with lactate, alanine or aspartate, had a greater glycogen loss than those incubated without substrate or with glycerol. Moreover, 10 mM quinolinic acid and 10 mM phenylpyruvate stimulated a greater glycogen loss when incubated with alanine and aspartate. In all incubation conditions, hepatocyte glycogen after a 1 hr incubation was always significantly below pre-incubation values (Table 6).

To eliminate the interference of endogenous glucose release on gluconeogenic rates, values were estimated by adding 1 μ Ci of each of the following: [U- 14 C]-lactate, [U- 14 C]-alanine, [U- 14 C]-aspartate and [U- 14 C]-glycerol. Simultaneously, the incorporation of these substrates was followed into radioactive CO_2 , lipid, and glycogen (Fig. 3). Of the four substrates, lactate, alanine and glycerol demonstrated the highest incorporation into glucose, whereas aspartate was essentially ineffective. Substrate incorporation into glycogen was very low compared with the other substances followed with the highest rate demonstrated by alanine. Lactate, glycerol and aspartate oxidation to CO_2 all approximated 100 nmoles per hr per 100 mg wet weight, but alanine was oxidized at a rate 7-times that of the other compounds. Incorporation into lipid was high for all four substrates and approximated the gluconeogenic rates except for aspartate where the rate was 4.4 fold greater.

Substrate incorporation into lipid was followed for two reasons firstly, to see if a reciprocal relationship exist between lipogenic

Figure 3. Gluconeogenesis in hepatocytes isolated from livers of fed St. Lawrence River eels. Livers were perfused and hepatocytes were incubated with the indicated radioactively labelled (1 μ Ci) substrates as described in Fig. 1. Control values represent the incubation of hepatocytes without inhibitors. These results were obtained from radioactivity measurements of the experiments reported in Table 6.

*Significantly different from control; t-test $P < 0.05$.



and gluconeogenic rates as reported in the mammalian liver (Nikkilä, 1973); and secondly, to locate the label to either the glycerol moiety or the fatty acid chain of the lipid fraction. Fig. 3 indicates that no reciprocal relationship exists between gluconeogenic and lipogenic rates under the conditions used in these experiments. Also, Table 7 provides strong evidence that all radioactivity in the lipid component is localized to the glycerol constituent and not the hydrocarbon chain of the fatty acids. Since glycerol-1-phosphate is derived from DHA-P (see Fig. I-1), a gluconeogenic intermediate, glycerol must be a product of the initial steps of gluconeogenesis. In further studies, radioactive substrate incorporation into lipid was followed as an indication of glycerogenesis through the gluconeogenic pathway.

To establish the path of gluconeogenesis, eel hepatocytes were incubated in the presence of the prior mentioned gluconeogenic inhibitors (Fig. 3). Quinolinic acid strongly inhibited gluconeogenic rates from lactate, alanine and aspartate by 52.5%, 33.5% and 61.1% respectively but, as expected, not from glycerol. Oxidation rates were not effected. Phenylpyruvate had a similar effect, except that the oxidation of alanine was significantly increased. Incorporation of substrates into glycogen was also inhibited by these two inhibitors, but the lack of significant difference could be a result of the low radioactive incorporation. In general, phenylpyruvate decreased radioactivity recovered as lipids (i.e., glycerol), but this was significant only for lactate.

Aminooxyacetate significantly inhibited alanine incorporation into CO_2 and glucose, but it had only a slight effect on aspartate incorporation (Fig. 3). A significant inhibition of alanine and

Table 7. The pattern of substrate incorporation into the glycerol and fatty acid components of eel hepatocyte lipids. Livers from fed St. Lawrence River eels were perfused and hepatocytes were incubated with the indicated radioactive (1 μ Ci) and cold substrates as described in Fig. 1. No differences were observed between the two lipid extraction procedures (see Methods and Materials) so results were combined. Results are expressed as the MEAN $\pm$ SEM and numbers in parentheses represent the total number of samples.

N.S. Lipid, non-saponifiable lipid.

ADDED SUBSTRATES	INCORPORATION OF SUBSTRATE INTO		
	GLYCEROL (nmoles of substrates/hr/100 mg)	FATTY ACIDS	N.S. LIPID
3 mM glucose	32.46 ± 1.52 (6)	0 (6)	0 (6)
1 mM lactate	20.67 ± 0.19 (12)	0 (12)	0 (12)
1 mM alanine	33.91 ± 1.57 (6)	0 (6)	0 (6)
1 mM aspartate	15.64 ± 0.57 (8)	0 (4)	0 (4)

aspartate incorporation into lipids (i.e., glycerol) is also observed.

B) Effects of cortisol on gluconeogenesis

The control of glucose release and glycogen depletion by cortisol was studied with 1 mM lactate as substrate during a 3 hr eel hepatocyte incubation (Fig. 4). Glucose released into the medium by the hepatocytes was linear during the entire incubation period, and 10 μ M cortisol decreased this release by approximately 10%. Glycogen depletion occurred at an initially rapid rate but fell slowly after 30 min. Ten μ M cortisol slightly, but not significantly, enhanced the glycogen depletion.

Gluconeogenic rates from [U- 14 C]-lactate remained constant during the first two hours of incubation, but declined by the third hour (Fig. 5A). These rates were significantly increased at all time periods by 10 μ M cortisol. The radioactivity in the glycogen fraction increased linearly during the first hour but decreased thereafter (Fig. 5 B). Cortisol significantly decreased this value only at the first hour. Oxidation of lactate followed a sigmoidal response which was not markedly altered by cortisol (Fig. 5 C). Radioactivity peaked in the lipid fraction by 30 min and remained constant thereafter; cortisol did not affect this pattern.

C) Effects of glucagon and dibutyryl-cAMP on gluconeogenesis

The effects of glucagon and dibutyryl-cAMP (a soluble derivative of cAMP) (Garrison and Haynes, 1973) were also investigated in the eel hepatocyte preparation. Incorporation into cellular constituents of radioactivity from 1 μ Ci [U- 14 C]-lactate and a mixture of amino acids (including 0.2 mM of each of alanine, aspartate, glutamate, glycine and leucine) to which was added 1 μ Ci of either [U- 14 C]-alanine or

Figure 4. Effects of cortisol on glycogen depletion (A) and total glucose release (B) in eel hepatocytes. Livers from three fed St. Lawrence River eels (fed about 5 months, starved 2 months, and fed 2 weeks prior to the experiments) were perfused and hepatocytes were incubated in duplicate with 1 mM lactate (1 μ Ci [U-¹⁴C]-lactate) as described in Fig. 1. Vertical bars represent the SEM of three separate experiments.

*Significantly different from control; t-test $P < 0.05$.

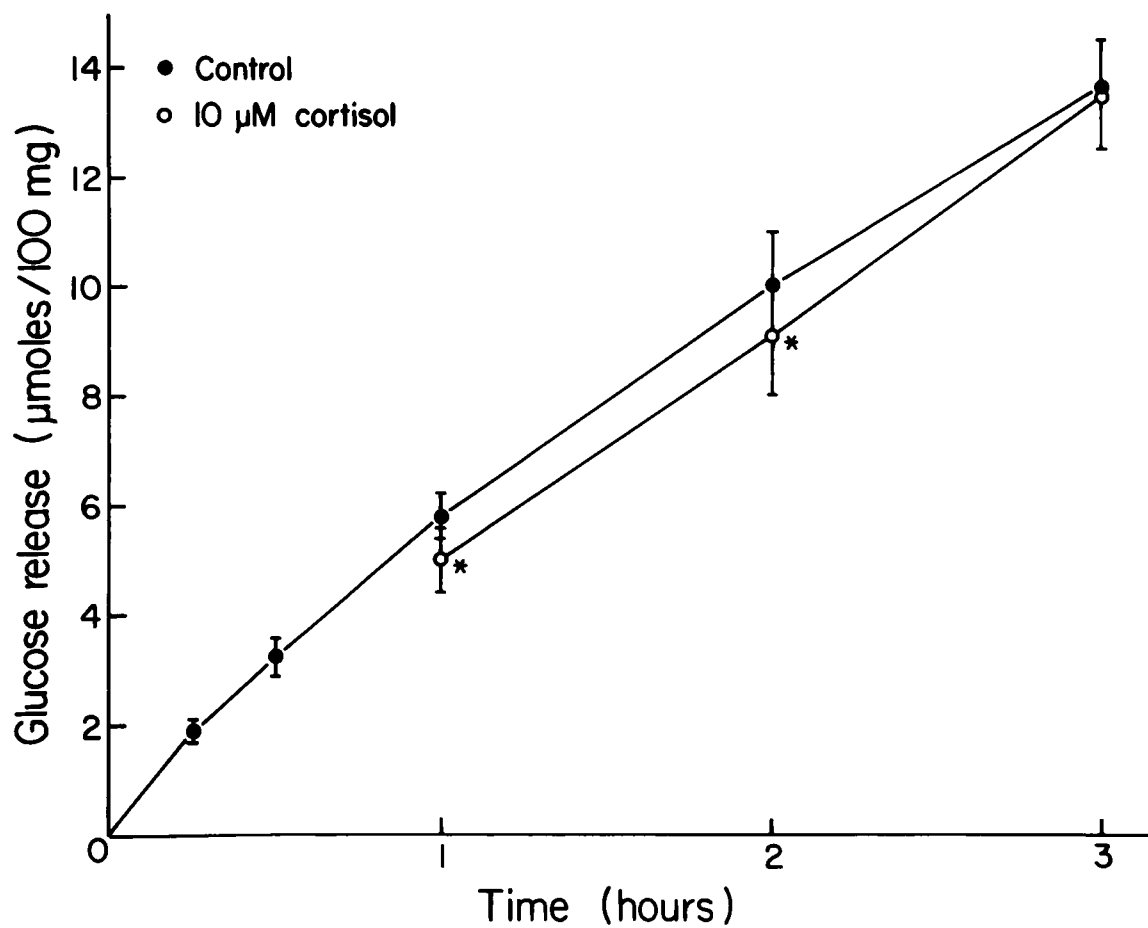
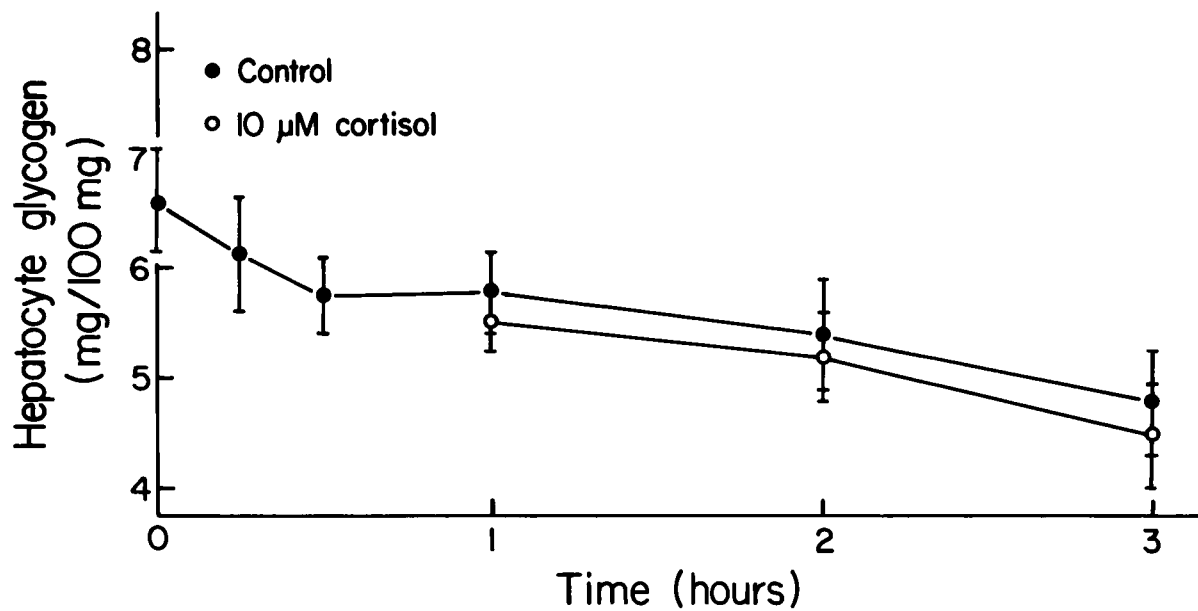
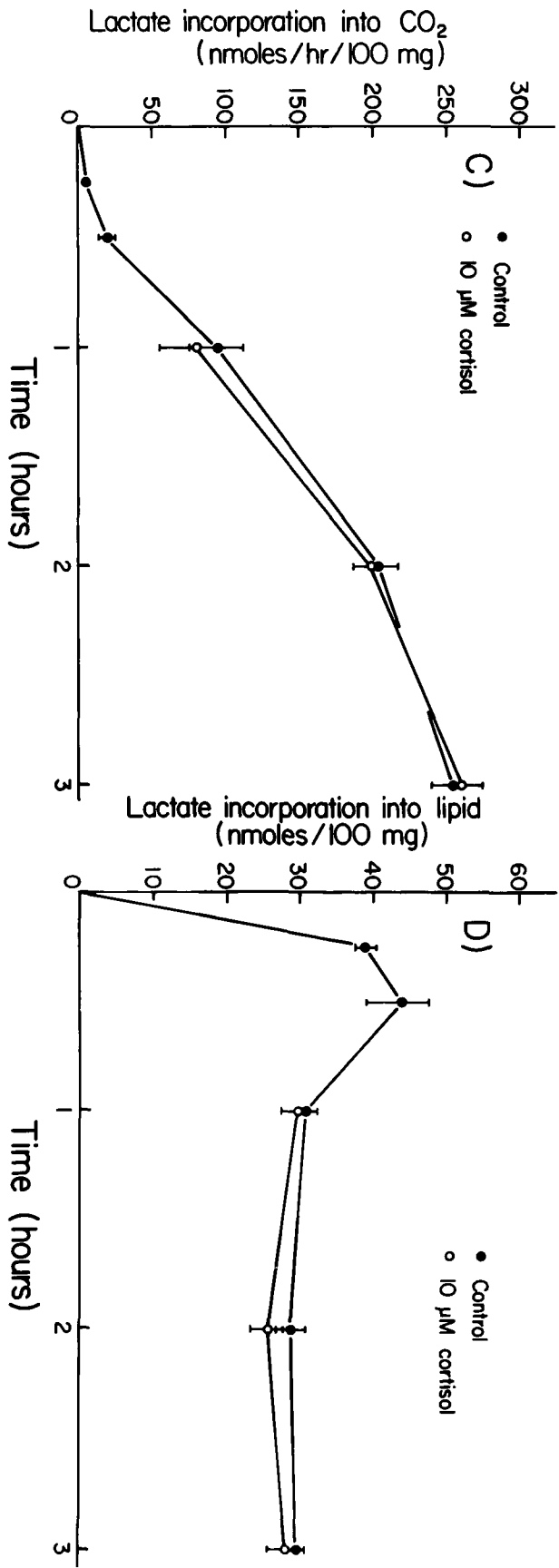
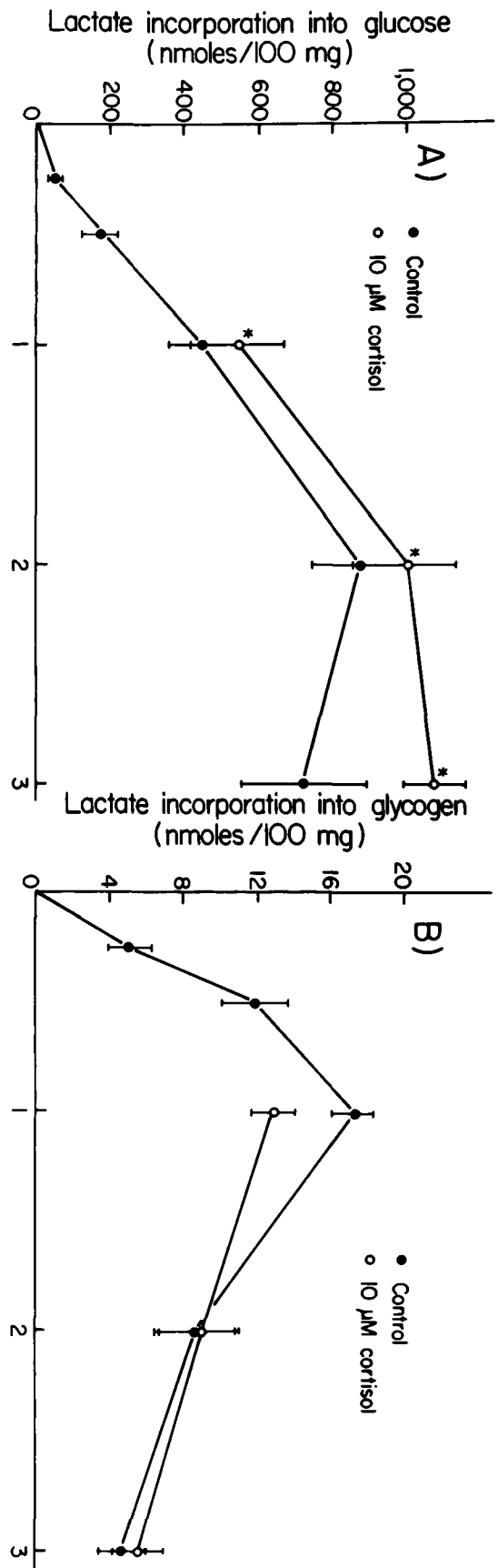


Figure 5. Effects of cortisol on the distribution of radioactive lactate into eel hepatocyte fractions. Those results were obtained from the same experiments shown in Fig. 4. Vertical bars represent the SEM of three individual fish. A) Lactate incorporation into glucose; B) Lactate incorporation into glycogen; C) Lactate incorporation into CO₂; D) Lactate incorporation into lipid.

*Significantly different from control; t-test $P < 0.05$



or [U-¹⁴C]-aspartate was followed (Figs. 6 and 7). Since the results of glucose release and glycogen loss for the two experiments were quite different, each experiment is reported separately; however, it should be noted that when compared to the controls, relative changes are very similar. Medium glucose was significantly increased by glucagon and dibutyryl cAMP regardless of the substrate used (Fig. 6). Glycogen depletion increased slightly in the presence of both glucagon and dibutyryl cAMP but the increase was less with dibutyryl cAMP.

Gluconeogenic rates did not differ between the two experiments so the results were combined. Rates were about 122, 60 and 9.2 nmoles per hr per 100 mg wet weight for lactate, alanine and aspartate, respectively (Fig. 7). Glucagon and dibutyryl cAMP significantly increased gluconeogenic rates from lactate, significantly decreased rates from aspartate but had no effect with alanine. Only lactate incorporation into glycogen decreased in the presence of glucagon and dibutyryl cAMP. Alanine incorporation into CO₂ and lipids (i.e., glycerol) was significantly decreased by both glucagon and dibutyryl cAMP. Neither oxidation nor lipid incorporation from lactate and aspartate were markedly altered by hormone addition.

Figure 6. Effects of bovine glucagon (14.7 μ M) and dibutyryl cAMP (0.1 mM diB cAMP) on glycogen depletion (A) and total glucose release (B) in eel hepatocytes. Livers from four fed St. Lawrence River eels were perfused and hepatocytes incubated as described in Fig. 1, except that the added substrates were 1 mM lactate or 1 mM amino acid mix (0.2 mM of each of alanine, aspartate, glutamate, glycine and leucine). Vertical bars represent the SEM of two fish in each experiment.

*Significantly different from control; t-test $P < 0.05$.

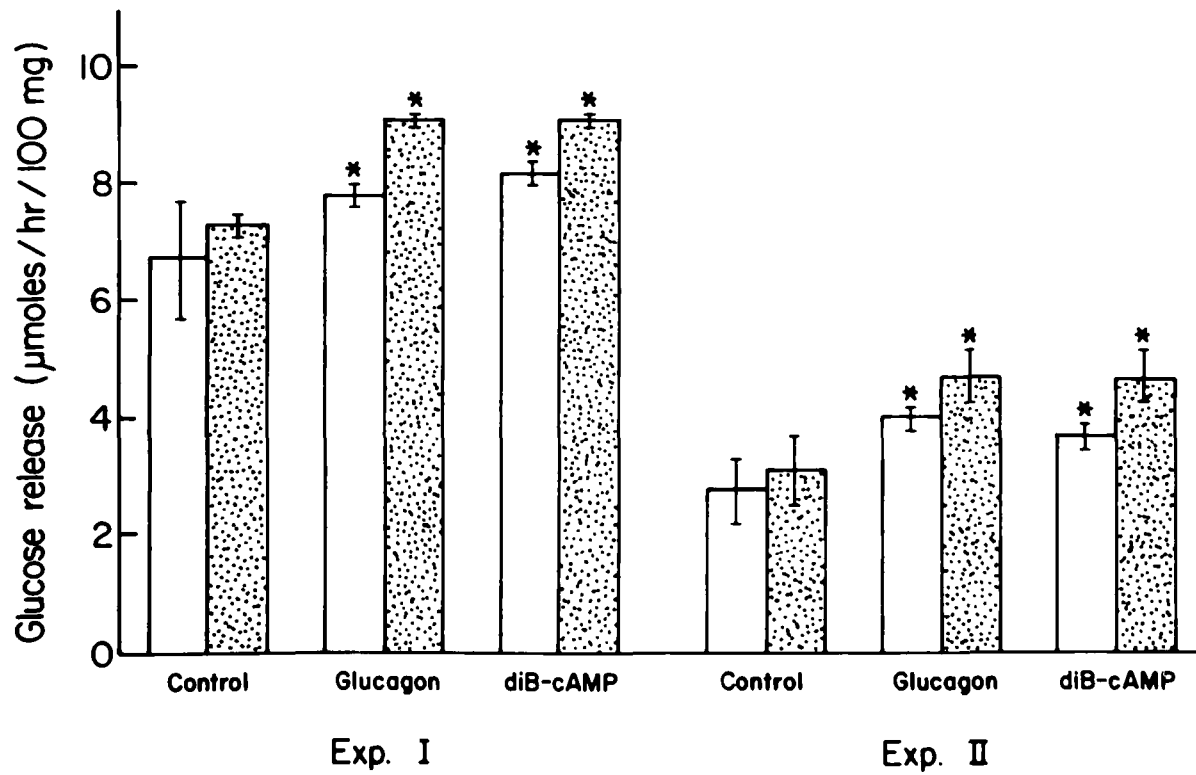
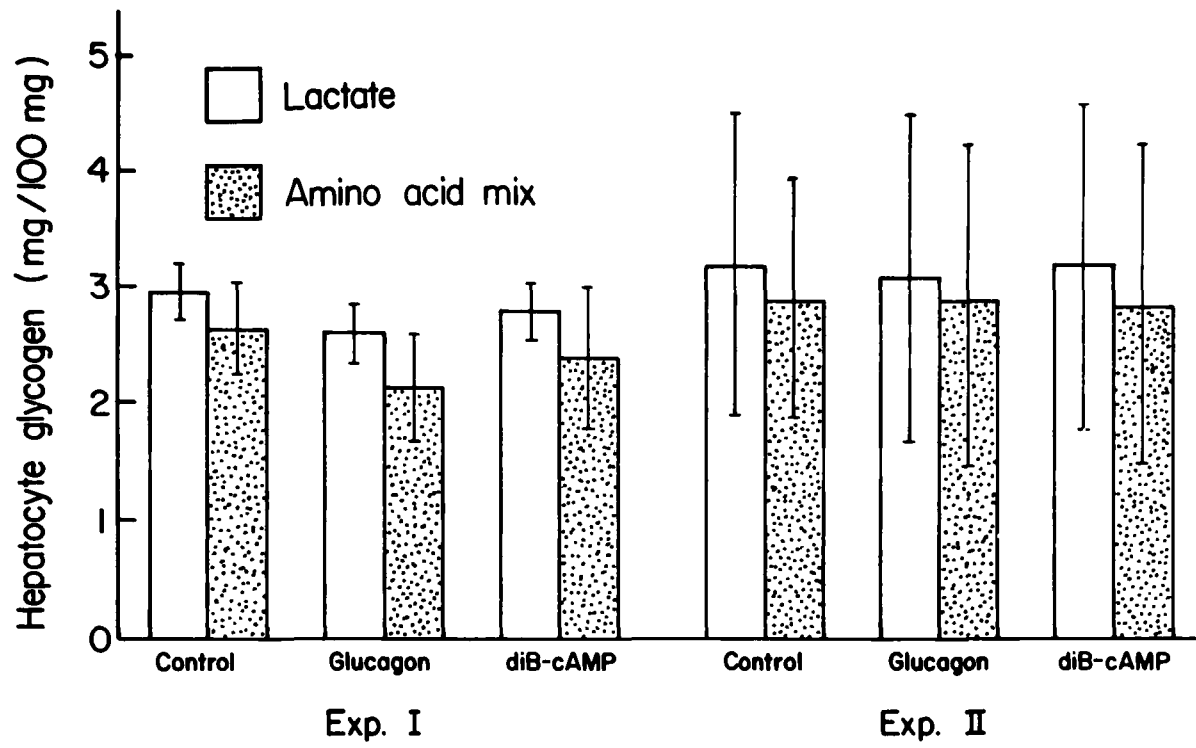
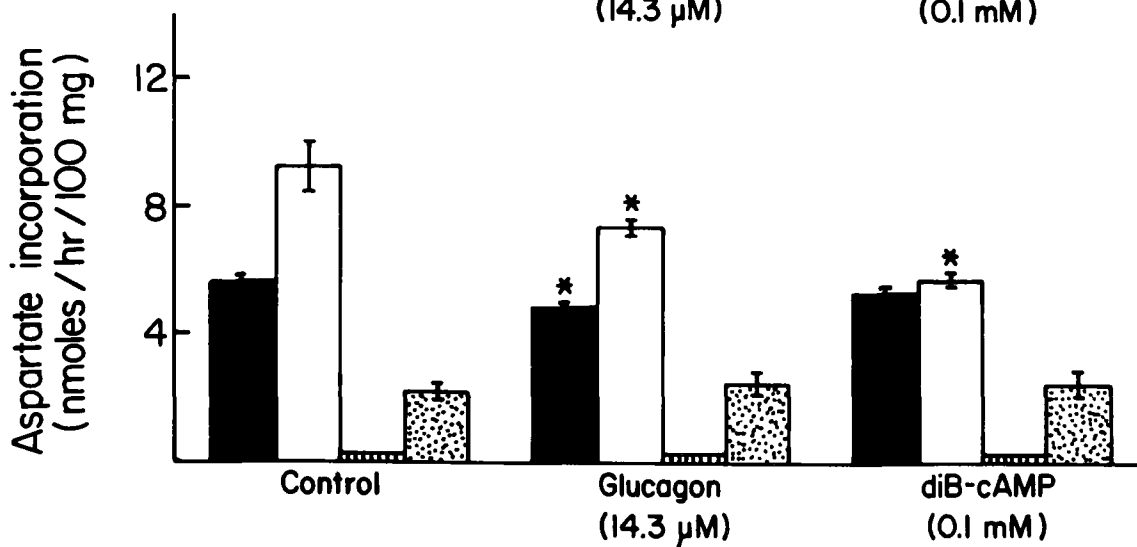
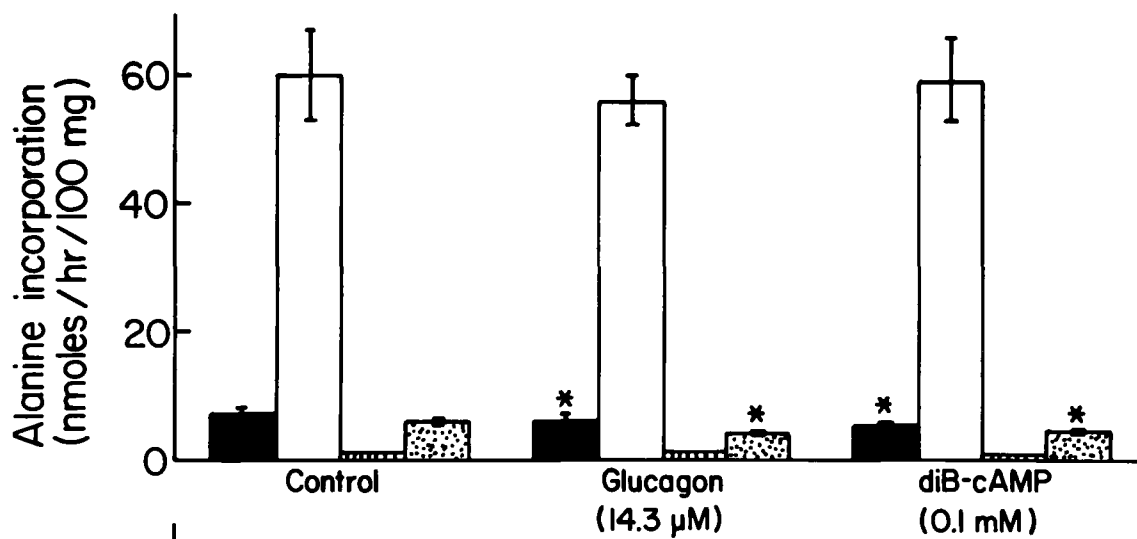
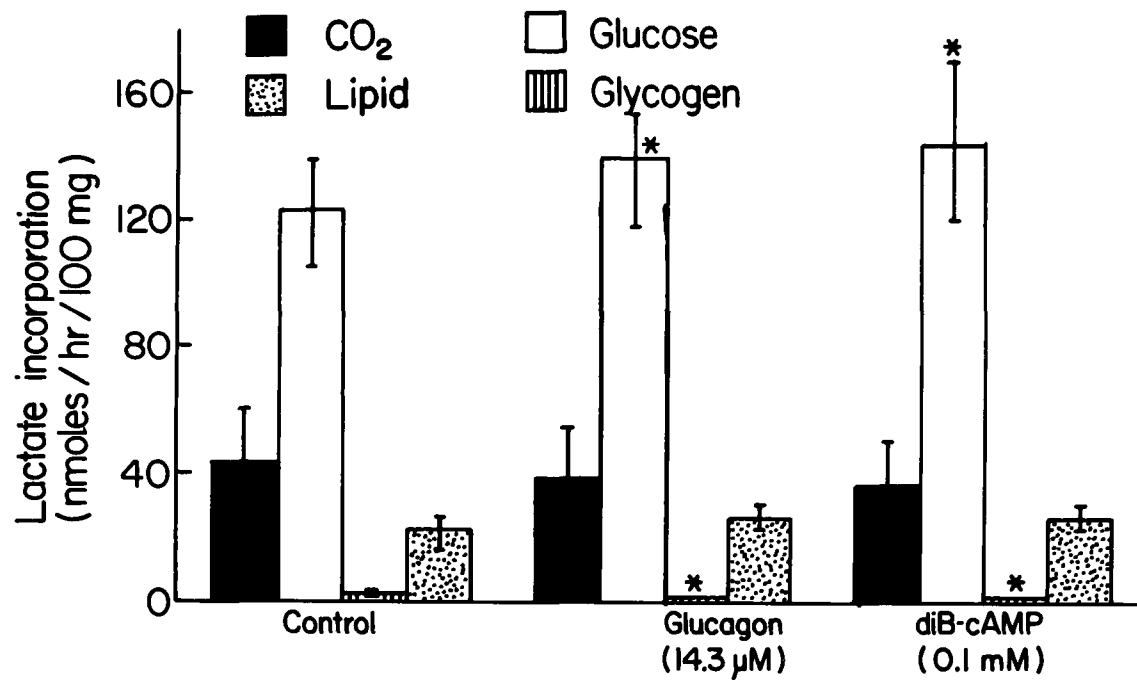


Figure 7. Effects of bovine glucagon (14.7 μ M) and dibutyryl cAMP (0.1 mM diB cAMP) on the distribution of radioactive lactate, alanine and aspartate into eel hepatocytes fractions. These results were obtained from the same experiment shown in Fig. 6. Radioactive additions were A) 1 μ Ci [$U-^{14}C$]-lactate B) 1 μ Ci [$U-^{14}C$]-alanine and C) 1 μ Ci [$U-^{14}C$]-aspartate. Vertical bars represent the SEM of four individual fish.

*Significantly different from control; t-test $P < 0.05$.



DISCUSSION

I. THE EEL HEPATOCYTE PREPARATION

A) Effects of calcium in the perfusate

The hepatocyte preparation has numerous advantages over other in vitro systems, but changes in cellular constituents occurring during the preparation could alter the metabolism of the hepatocytes to such an extent that it could differ totally from the in vivo situation. As in the mammalian preparation, the eel hepatocyte differs from the whole liver principally in glycogen and ionic compositions when livers were perfused with a Ca^{++} -free perfusate (Table 1).

A short perfusion with a Ca^{++} -free perfusate followed by a 1 or 4 mM Ca^{++} -containing perfusate reduced the required time of perfusion and resulted in a much better rat hepatocyte preparation (Seglen, 1972). Ca^{++} addition to the eel perfusate presented a similar situation. There are, however, two disadvantages of Ca^{++} -perfusion in the eel liver. First, red blood cell contaminations increase and, second, an enhanced Na^+ gain (Table 1 and 2). Numerous advantages were also observed. Cell membranes appear more stable in both groups of fish as judged by trypan blue exclusion. No significant changes were observed in the cellular K^+ and Ca^{++} content. And, respiration rates were 30% higher in hepatocytes prepared with Ca^{++} than without it.

Whereas some experiments used a preliminary Ca^{++} -free perfusion step, all gluconeogenic studies used hepatocytes prepared with a 1 mM Ca^{++} -containing perfusate medium.

B) Effects of Ca^{++} , glucose, albumin and gelatine in hepatocyte incubations

Incubation of hepatocytes presents two major problems. The

first is the choice of a suitable physiological medium. A teleost saline buffer with phosphate rather than bicarbonate was used to avoid saturation of the CO_2 trapping medium during incubation. Eel plasma contains about 5.0 g protein per 100 ml (Larsson and Fange, 1969; Larsson and Lewander, 1972), but these media do not contain any protein or other biologically significant substances such as hormones. Secondly, Ca^{++} , which is an integral part of ringer solution, increased cell clumping (Seglen, 1972; Berry, 1975), causing problems when the cell suspension was divided into individual incubation flasks. Therefore, to optimize the incubation medium, experiments were carried out using a Ca^{++} -free phosphate medium, and media containing either 0.2% albumin (Krebs et al., 1973) or 1.5% gelatine (Katz et al., 1975).

The absence of Ca^{++} from the incubation medium was deleterious to the hepatocytes. The hepatocytes were less stable since the percentage of hepatocytes which excluded trypan blue and the total number of cells decreased, especially with added albumin (Table 3). The amount of protein released into the medium increased and a major disturbance in cellular ionic concentrations was observed which continued throughout the incubation period. Such disturbances, and especially that seen for the ions, may be partially responsible for the 50% increase in oxygen uptake (Table 3). Albumin addition reversed this increase in oxygen uptake. The presence of albumin in the Ca^{++} -free incubation medium and the absence of Ca^{++} from the perfusion medium both resulted in a loss of hepatocyte Ca^{++} (Tables 1 and 3). A decrease in cytosolic Ca^{++} may shift Ca^{++} from mitochondria. Mammalian mitochondria require Ca^{++} for stability and the maintenance of oxygen uptake (Carafoli, 1975; Scarpa, 1975). Assuming that eel mitochondria

behave in a similar manner, an absence of Ca^{++} could have lowered the oxygen uptake and, therefore, Ca^{++} was present in all further experiments.

The addition of albumin to the standard Ca^{++} -phosphate medium had no significant effect on hepatocyte properties. Similarly, the addition of 1.5% gelatine did not significantly influence the hepatocytes except that Na^+ concentrations increased during the incubation period (Table 4). Interestingly, both albumin and gelatine enhanced the glucose stimulation of oxygen uptake. The metabolism of $[\text{U}-^{14}\text{C}]\text{-glucose}$ was studied in the presence of these proteins to elucidate these respiratory rate effects. When eel hepatocytes were incubated in the standard phosphate buffer, a direct relationship between glucose uptake and medium glucose existed, while glucose release and turnover were essentially zero. However, the addition of either protein stimulated glucose release and uptake leading to a higher glucose turnover rate. This higher turnover is certainly not energetically advantageous and may help to explain the increased O_2 uptake when glucose was added to the hepatocytes in the presence of either albumin or gelatine. While respiration rates increased, oxidation of $[\text{U}-^{14}\text{C}]\text{-glucose}$ decreased in the presence of albumin, but no trend was observed with gelatine. Thus, the increased O_2 uptake may be exaggerated by a higher medium glucose turnover, but glucose itself is not necessarily the major metabolic fuel.

Since St. Lawrence River eel hepatocytes were incubated with $[\text{6-}^{14}\text{C}]\text{-glucose}$ instead of $[\text{U}-^{14}\text{C}]\text{-glucose}$, incorporation rates of glucose into CO_2 , lipid and glycogen cannot be easily compared. It can be tentatively concluded that glucose uptake by the liver cells is enhanced with eel maturation, although the exact fate of this glucose remains obscure. This statement is based upon: 1) both the net glucose

uptake (from enzymatic determination) and the apparent glucose uptake (from radioactive determination) of the hepatocytes prepared from the Ottawa River eels was about 25% of the added medium glucose while the apparent glucose uptake of hepatocytes from the smaller and younger eels i.e., those from St. Lawrence River, was less than 1% of the medium glucose; and 2) at 3 mM glucose, these hepatocytes still released glucose into the medium, and only insulin or 10 mM glucose addition prevented this behaviour (Table 5).

The relatively small utilization of glucose by the younger eel hepatocytes is fortunate since a large uptake of glucose will underestimate gluconeogenic rates. Since albumin and gelatine enhance glucose uptake, neither of these proteins were included in the incubation medium for further experiments.

C) Energy charge

Energy charge, or the average number of anhydride phosphate bonds per adenosine moiety (Atkinson, 1977), is an indicator of the energy state of the cell. A value of 0.8-0.9 is normal for viable cells (see Atkinson, 1977), and since this value was maintained throughout the incubation period for the eel (Fig. 2 B) no major disturbances in energy metabolism would be expected. Similar results have been reported for the rat liver system (Krebs et al., 1973). Concentrations of G-6-P and pyruvate, two glycolytic intermediates, were always found above the normal in vivo liver concentrations (Fig. 2 A). The pre-perfusion increase may have resulted from a momentary anaerobic condition imposed on the liver during anaesthesia. Fortunately, these concentrations decreased during the perfusion indicating that perfusate oxygenation was adequate. Since glucose oxidation was low and pyruvate

concentrations remained constant during a 2 hr incubation, increases in G-6-P concentrations, at 1 hr of incubation may have been from glycogen breakdown.

Therefore, hepatocytes prepared from livers of St. Lawrence River eels perfused with a Ca^{++} -containing bicarbonate buffer, pH 7.4, and incubated for up to 3 hrs in the standard Ca^{++} -phosphate buffer, pH 7.4, maintained their viability and metabolite contents similar to whole liver values. Thus, metabolic studies with such eel hepatocytes approximated conditions existing in an in vivo situation.

II. GLUCONEOGENESIS IN EEL HEPATOCYTES

A) Glucose release

Depending upon the physiological condition of the liver cells (i.e., glycogen storage levels, hormones, etc.), glucose derived from glycogen may be very important in the maintenance of blood glucose. In the absence of substrates, fed rat hepatocytes release 4 to 7 μmoles glucose per hour per 100 mg wet weight at 37 °C (Exton et al., 1970; Garrison and Haynes, 1973; Phillips and Hird, 1977). This endogenous release has been shown to be derived from storage glycogen in rabbit liver slices (Sutherland et al., 1965) and in rat hepatocytes (Garrison and Haynes, 1973).

Total glucose released by fed eel hepatocytes was linear for at least 3 hrs (Fig. 4) at a rate of about 6.0 μmoles per hr per 100 mg wet weight (Table 6), a rate which is similar to that reported by Phillips and Hird (1977) for the Australian eel, A. australis.

In both eel species it appears that this endogenous glucose release is associated with glycogen breakdown, so presumably

glycogenolysis is occurring. In fact, about 8.6 μ moles glucose equivalents per 100 mg wet weight were produced from glycogen breakdown after a 1 hr incubation (Table 6). The glucose released into the medium represented 70% of that glucose produced, with the additional 30% remaining within the cell, as reported for the fed rat hepatocyte (Garrison and Haynes, 1973). Since these eel hepatocytes were incubated without substrates, a fraction of this 30% would presumably be oxidized for ATP production while another fraction would be converted to glycerol (Table 5 and 7).

In the fed rat hepatocyte, addition of 10-20 mM lactate increases glucose release by 1.1 to 2-fold (Exton et al., 1970; Phillips and Hird, 1977). In fed American eel hepatocytes, however, addition of 10 mM of either lactate, glycerol or alanine significantly decreased total glucose release; aspartate, a poor gluconeogenic substrate, had no effect (Table 6). These results contrast those reported for the Australian eel liver slices (Phillips and Hird, 1977) and the perfused Japanese eel liver, A. japonica (Hayashi and Ooshiro, 1975). On the other hand, when results obtained from these three species are compared, they differ on many points of view, and therefore these results may reflect different adaptations to different environment.

Initially, it may be proposed that these substrates play a glycogen sparing role in the American eel hepatocyte. Addition of 10 mM lactate or alanine, however, enhanced glycogen breakdown while glycerol had no significant effect (Table 6). This increase in glycogen breakdown may have resulted from a low glucose concentration in the incubation medium. In the mammalian liver, glucose regulates its own release and uptake as well as glycogen synthesis and degradation. The latter

effect is through a direct glucose activation of glycogen phosphorylase phosphatase to decrease glycogen phosphorylase "a" activities and reciprocally activate glycogen synthetase (Hers et al., 1973; Hue, 1975; Walli and Shimassek, 1975). Whereas a good gluconeogenic substrate, such as lactate or alanine, can provide glucose for export (Fig. 3), glucose, per se, would have to be present to prevent glycogen breakdown. To explain a decrease in glucose release, these substrates or a direct gluconeogenic intermediate of these substrates must simultaneously inhibit glycogen breakdown to glucose, but at a different metabolic step than glucose inhibition. This possible inhibition by gluconeogenic substrates and/or intermediates was shown by the fact that in the presence of 3 mM glucose, eel hepatocytes still released 2.22 μ moles glucose per hr per 100 mg wet weight into the incubation medium. The role of a metabolic intermediate on the control of glycogen metabolism has recently been proposed in frog liver (Castineiras et al., 1978).

Two questions remain to be answered: how does glycerol, a good gluconeogenic substrate, prevent glycogen loss even in the absence of medium glucose and how does aspartate, a poor gluconeogenic substrate, enhance glycogen breakdown? As with lactate and alanine, the presence of glycerol leads to enhanced gluconeogenesis and glucose availability for export (Fig. 3). Apart from its gluconeogenic role, it is apparent that glycerol must inhibit a step between glycogen and G-6-P or a step between glycogen and glycerol since glucose is rapidly metabolized to glycerol (Fig. 1, Table 5); i.e., glycerol may be functioning in the system as does glucose in the mammalian glycogen phosphorylase system. Interestingly, Soeling et al. (1978) have reported that high glucose

concentrations activates PFK phosphatase which ultimately activates PFK. Inversely, glycerol may inhibit PFK activities in eel hepatocytes, and thus prevent glucose (or G-6-P) conversion to glycerol. This inhibition would prevent both glucose and glycerol production from glycogen. Thus, in the eel hepatocyte, both high glucose and high glycerol may prevent glycogen breakdown.

Aspartate has relatively little effect on glucose release (Table 6, Fig. 3), but its rate of incorporation into glycerol is similar to the other substrates (Table 7, Fig. 3). Therefore glycogen flux to glucose is not reduced, but the amount of glycerol produced from aspartate is ineffective in preventing glycogen breakdown, resulting in glycogen depletion. The important question is why does aspartate not produce glucose in appreciable amounts if it can produce glycerol? Further work must be done to answer this question.

Even though no exact mechanism can now be proposed it seems that a good gluconeogenic substrate, such as lactate, glycerol or alanine, inhibits glucose production from glycogen while a good glycerogenic substrate, such as lactate, alanine or aspartate, probably inhibits glycerol formation from glycogen. Glucose and glycerol, however, have to be present in order to inhibit excessive glycogen breakdown.

B) Gluconeogenic rates in eel hepatocytes

In the fed rat liver, rates of gluconeogenesis (corrected for glycogenolysis) from substrates, such as lactate, alanine, glycerol, serine and proline, vary between 2 to 3 μ moles glucose per hr per 100 mg wet weight at 37°C (Ross et al., 1967; Exton et al., 1970; Felig and Wahren, 1974), while insignificant rates are observed from glutamate and aspartate. In this study, addition of 10 mM of either lactate,

alanine or glycerol gave approximately 0.40, 0.60 and 0.90 μ moles glucose per hr per 100 mg wet weight at 15°C. As in mammals, synthesis of glucose from aspartate was not significant. Thus, gluconeogenic rates are lower in eel hepatocytes than in rat hepatocytes, but the eel liver is still able to release glucose in vitro at a rate similar to that of the rat liver from a combination of gluconeogenesis and glycogenolysis. Similar values have been reported for lactate gluconeogenesis in liver slices from the Australian eel by Phillips and Hird (1977), but their values for the rat are well below those values found in the literature.

The key gluconeogenic enzymes, PEP CK and PC, have been reported in eel liver (Phillips and Hird, 1977) and their activities appear sufficient to support the gluconeogenic rates observed in this study. The presence of a particular enzyme gives only circumstantial evidence of its importance in a particular metabolic sequence. This study, however, incubated eel hepatocytes in the presence of specific inhibitors of the gluconeogenic enzymes (Williamson et al., 1973). If lactate, alanine and aspartate flux through PEP CK as in rat liver, gluconeogenesis from these substrates should be inhibited by quinolinic acid while no change should be observed from glycerol since it arises at the level of dihydroxyacetone (DHA) in the gluconeogenic pathway, i.e. after PEP CK and PC (See Fig. I-1).

These predictions were substantiated (Fig. 3), but interestingly, quinolinic acid did not significantly alter the label incorporated into the glycerol moiety of the lipid (Fig. 3). This unexpected paradox can not be explained. Phenylpyruvate did reduce incorporation into glycerol but this was significant only from lactate. Oxidation of alanine was enhanced in the presence of phenylpyruvate, which indicates carbon flux

is shifted away from gluconeogenesis towards the Krebs' cycle by this inhibitor. As expected, aminooxyacetate significantly reduced the metabolism of alanine and aspartate into all assayed fractions. These results demonstrate that eel liver gluconeogenesis proceeds along a path similar if not identical to the rat gluconeogenic pathway and that amino acid transaminases are important as a first enzymic step in amino acid utilization.

C) Substrate incorporation into CO₂, lipid and glycogen

Substrate oxidation is of minor importance to the metabolism of lactate, glycerol and aspartate in the eel hepatocyte (Fig. 3). Only alanine produced significant CO₂, and its oxidation rate was 7-times greater than lactate, aspartate and glycerol. Incorporation of these substrates into glycogen was a minor metabolic route. Similar observations were made from glycerol in lamprey liver (Savina and Wostczak, 1977), from alanine in trout liver (Cowey *et al.*, 1977 a), and from glycerol, lactate and glycine in rat liver (Kendysh and Moroz, 1972).

An important metabolic route for these four substrates was their incorporation into the lipid soluble fraction. Studies with Northern pike should show that ¹⁴C-glycine incorporation into lipid is equivalent to that into proteins (Ince and Thorpe, 1976b). In this study, lactate incorporation into lipid reached equilibrium after 15 min of incubation and remained constant for up to 3 hrs (Fig. 5). The incorporation of substrate into the lipid fraction was through glycerol not the fatty acid chain (Table 5). This result agrees with the lack of malic enzyme and citrate cleavage enzyme activities in eel liver (Aster and Moon, 1977). Acetate does represent a good *in vivo* lipogenic substrate in the American eel, but its precise rate and pathway of incorporation

is only now being investigated (Aster, unpublished). Under the incubation conditions reported here, fatty acid synthesis was limited by the absence of suitable substrates and thus incorporation into glycerol would saturate quickly in the absence of free fatty acids for esterification. Even with glycerol addition, no significant difference in lipid incorporation was noted compared to that observed from lactate, alanine and aspartate. Shrago et al (1978) have reported that the amount of glycerol produced from lactate or pyruvate and incorporated into rat adipocyte lipid exceeds the amount of fatty acid synthesized and incorporated into lipid. Therefore, a fraction of glycerol incorporated into eel hepatocyte lipid could be due to a high turnover of glycerol in this lipid fraction.

These results indicate: 1) any mitochondrial acetyl CoA produced from these substrates is oxidized to CO_2 and H_2O and does not participate in lipogenesis; 2) pyruvate produced from these four substrates is either oxidized or enters into the gluconeogenic pathway; and 3) glycerogenesis from lactate and alanine occurs through the PEP CK and PC steps, so these two enzymes are involved in both lipogenesis and gluconeogenesis. Since both absolute rates of lipogenesis and gluconeogenesis are high, the reciprocal relationship which has been reported between these two pathways in the rat liver (Nikkila, 1973) would be absent in the eel liver. A similar conclusion was reached by Ince and Thorpe (1977 a) in the Northern pike. To postulate a role for PEP CK and PC in glycerogenesis, the apparent lack of inhibition of glycerol production from lactate and alanine by quinolinic acid and phenylpyruvate must be explained. Three possibilities exist: 1) the synthesis of glycerol is by another metabolic route, but this seems

unlikely since phenylpyruvate had some inhibitory effects; 2) the competition for DHA by aldolase and glycerophosphate dehydrogenase would favor the latter enzyme due to a kinetic advantage; and 3) the availability of fatty acids limits glycerol incorporation into lipids. In this latter case, only a fraction of the total glycerol-1-phosphate produced is incorporated into lipids, and the inhibitors may not reduce the concentration of this compound below the critical level necessary to observe a change. The last two possibilities could explain this result, and present experiments are examining this problem.

D) Cortisol control of gluconeogenesis

Cortisol has a similar gluconeogenic role in eel liver as in rat liver. Total glucose released was significantly inhibited by cortisol (Fig. 4), which might be explained by a glycogen sparing effect as reported in mammals (Kendysh and Moroz, 1972). Although there was a tendency for glycogen values to drop in the hepatocyte system with cortisol (Fig. 4), Butler (1968) has reported an enhanced eel liver glycogen synthesis in vivo after cortisol injection. Also, cortisol does significantly stimulate lactate gluconeogenesis, and as explained previously, the lack of glucose in the incubation medium could have prevented glycogen sparing. It has been reported that net glycogen synthesis in the rat hepatocyte occurs only if non-physiological glucose concentrations (25-30 mM) and gluconeogenic substrates are added to the medium (Walli, 1977). Since glucose was not added, it must be considered to be a factor together with other possible components.

Interestingly, cortisol is known to increase PEP CK activities in mammals (Krone et al., 1976), yet there was no increase in lactate incorporation into eel hepatocyte lipid. Again, this may be due to

limitation in availability of the free fatty acids or that cortisol specifically increases gluconeogenesis by increasing the flux of DHA to glucose without affecting directly the flux of DHA to glycerol. The two key gluconeogenic enzymes FDPase and G6Pase could be involved in this process.

E) Glucagon control of gluconeogenesis

As with cortisol, glucagon behaves in an analogous manner in eel hepatocytes as in the rat liver system; stimulation of glucose release, stimulation of lactate gluconeogenesis and inhibition of glycogen synthesis from all substrates (Garrison and Haynes, 1973). Also, cAMP appears to be involved in the action of glucagon since dibutyryl cAMP had similar effects. However, two major differences exist between the rat and the eel. Firstly, glucagon and dibutyryl cAMP were unable to increase glycogen breakdown significantly. This is in agreement with the report of Inui and Yokote (1977) in glucagon-injected Japanese eels. Secondly, gluconeogenesis increases only from lactate in the presence of glucagon and dibutyryl cAMP. Whereas at high concentrations alanine is a good gluconeogenic substrate (Fig. 3) at low concentrations it is less effective than lactate (Fig. 7) and gluconeogenesis is not stimulated by glucagon and dibutyryl cAMP from this substrate.

Glucagon had some effect on alanine incorporation into CO₂ and lipids and aspartate incorporation into CO₂ and glucose, but these are minor since rates were initially very low. The precise mechanism of glucagon activation of gluconeogenesis is not known, although Murat et al. (1978) suggest PEP CK may be involved.

Consequently, eel liver appears to be an important organ for blood glucose homeostasis. In a recent study, Inui and Yokote (1977) observed

no glucose in blood samples taken from the bulbus arteriosus of an hepatectomized Japanese eel suggesting the liver is the principal organ involved in glucose release. The source of this glucose may be either liver glycogenolysis or gluconeogenesis with the balance between these two processes being determined by hormones, substrates, etc. Glycogenolytic rates in eel hepatocytes are similar to those reported in rat hepatocytes. Total glucose released from the glycogen stores is inhibited by good gluconeogenic substrates (e.g., lactate, alanine and glycerol) even though the gluconeogenic rates in eel hepatocytes were lower than those in rat hepatocytes. Unlike mammals, glucagon increases gluconeogenic rates only from lactate not from alanine and even though the total glucose release by eel hepatocytes is higher with glucagon, no significant changes in glycogen depletion are observed. Cortisol does increase lactate gluconeogenesis but inhibits total glucose release by 10%. Thus, some major similarities exist between the mammalian and eel hepatocyte system, but it is possible that the differences noted in this paper are exaggerated during long term starvation in the American eel.

IV

GLUCONEOGENESIS AND STARVATION IN THE
AMERICAN EEL HEPATOCYTE

INTRODUCTION

Liver glycogen stores are totally depleted after a 48 hr fast in the rat (Steiner and Williams, 1959; Newsholme and Start, 1976) and gluconeogenic rates from lactate and amino acids are enhanced (Ross et al., 1967a, b; Johnson et al., 1972; Walter, 1973) to provide adequate blood glucose for the maintenance of nerve tissue integrity and other tissues which rely totally on glucose for energy (Cahill, 1976). Skeletal muscle proteolysis and amino acid release are also enhanced during starvation to provide an adequate supply of gluconeogenic substrates (Felig and Wharen, 1974; Karl et al., 1976).

Fish, however, are adapted to extensive starvation periods as would be encountered during a low temperature fast or an extensive non-feeding spawning migration (see review by Woodhead, 1975). Unlike mammals, liver glycogen stores remain relatively constant during starvation in many fish; e.g., values in the goldfish, Carassius auratus, were unchanged after 25 days of starvation (Stimpson, 1965); values in the Northern pike, Esox lucius (Ince and Thorpe, 1976a) and the rainbow trout, Salmo gairdneri (Cowey et al., 1977a), were about 50% of those observed in fed animals; and in the European eel, Anguilla anguilla, a significant depletion was observed only after 5 months of starvation at 5 °C (Larsson and Lewander, 1973; Dave et al., 1975). While carbohydrate stores are relatively constant during starvation, blood glucose values tend to drop in Northern pike (Ince and Thorpe, 1976a) and rainbow trout (Cowey et al., 1977a), but no consistent trend was observed in the European eel (compare results of Larsson and Lewander, 1973, with Dave et al., 1975).

Cowey et al. (1977b) proposed that glycogen is not serving as an immediate source of blood glucose and that there is a very low exchange

between glycogen and glucose. Moreover, many investigators have suggested, but not provided evidence for, an increase in liver gluconeogenesis from amino acids during starvation in fish to maintain both blood glucose and body carbohydrate stores (Butler, 1968; Dave et al., 1975; Cowey et al., 1977b). Cowey et al. (1977a) have reported, however, that glucose production from pyruvate in rainbow trout liver slices was approximately 10-times lower than that reported in rat liver and that no differences existed in alanine gluconeogenesis between rainbow trout which were fasted or fed a high protein diet. Gluconeogenic rates from lactate in perfused livers of the Japanese eel, Anguilla japonica, were similar to those in rat liver, but there was no correlation between the glucose production and the duration of starvation (Hayashi and Ooshiro, 1975). In Chapter 3 of this thesis, poor gluconeogenic rates were observed from physiological concentrations of alanine and aspartate, and glucagon did not enhance these rates as has been reported in mammals (Mallette et al., 1969). Therefore, the exact role of gluconeogenesis during starvation in fish remains unknown.

Glucose utilization has been demonstrated to be very low in the plaice, Pleuronectes platessa (Cowey et al., 1975) and in the carp, Cyprinus carpio (Creach and Serfaty, 1974). Also, these latter investigators found a lower glucose oxidation in starved than fed carp which agreed with the study of Beamish (1964) which showed that O_2 uptake was lower in starved than fed white sucker and rainbow trout.

A low glucose utilization may indicate that other metabolites, such as triglycerides and proteins, could be important energy sources. Creach and Serfaty (1974) have demonstrated severe protein depletion in carp liver, muscle, kidney, intestine and heart after a winter

starvation. Fat stores were totally depleted after the non-feeding spawning migration of the Fraser River sockeye salmon (Idler and Bitners, 1958a,b). In the European eel, a large depletion in liver and muscle lipid was observed during the first three months of starvation while skeletal muscle protein content gradually, but not significantly, decreased over a 5 month starvation period (Dave et al., 1975). Therefore, carbohydrate stores and blood glucose in fish can be maintained during starvation either by increases in gluconeogenesis and/or increases in the mobilization of lipids and proteins for energy production which decreases glucose demands.

In this study, changes in gluconeogenic rates were compared with changes in tissue metabolite concentrations during a 10 month starvation period at 5° and 15° C in the American eel, Anguilla rostrata. Gluconeogenic rates were estimated in isolated eel hepatocytes as described in Chapter 3 using physiological concentrations of lactate and the two amino acids alanine and aspartate. Physiological concentrations of substrates were used since these occur in vivo during starvation and gluconeogenic rates will more closely approximate those which might occur in the living animal. These results indicated that although a significant depletion of muscle protein occurred during starvation, lactate gluconeogenesis exceeded both alanine and aspartate gluconeogenesis during most of the starvation period at 5° C and 15° C.

RESULTS

I. EFFECTS OF STARVATION ON TISSUE METABOLITES

American eels in our laboratory conditions did not feed below 10°C. This is consistent with behavioral studies reported by Swedish workers for the European eel (Nyman, 1972). Therefore, starvation at 5°C should be considered as a natural phenomenon occurring in the environment of the American eel. Metabolic changes concurrent with this starvation period may have been due to normal seasonal changes. To reduce seasonality problems, metabolites and gluconeogenic rates were measured in eels fed a beef liver diet at 15°C over the same time period. Since the two groups of fish were held at two different temperatures, a third group of eels was starved at 15°C. In the fed group held at 15°C, no significant differences were noted in metabolites or gluconeogenic rates during the 10 month experiment so all values were grouped as time 0 controls. Since the 5°C held eels did not feed, the 15°C control value was assumed for the 5°C experimental group.

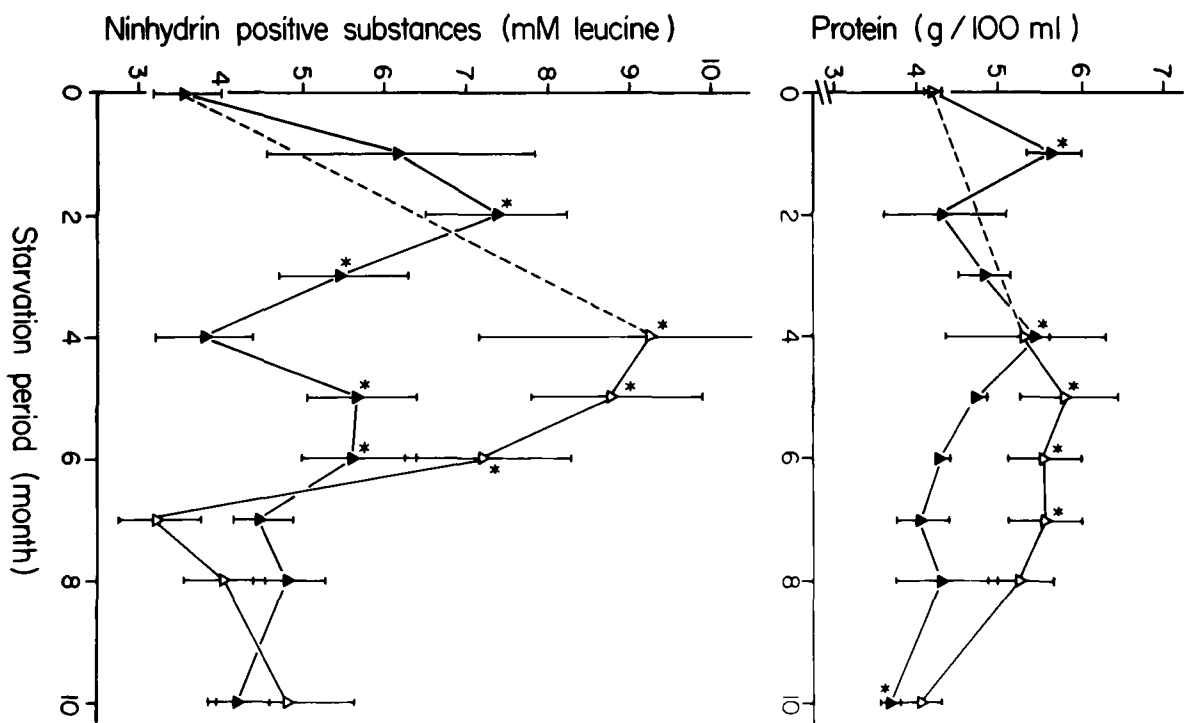
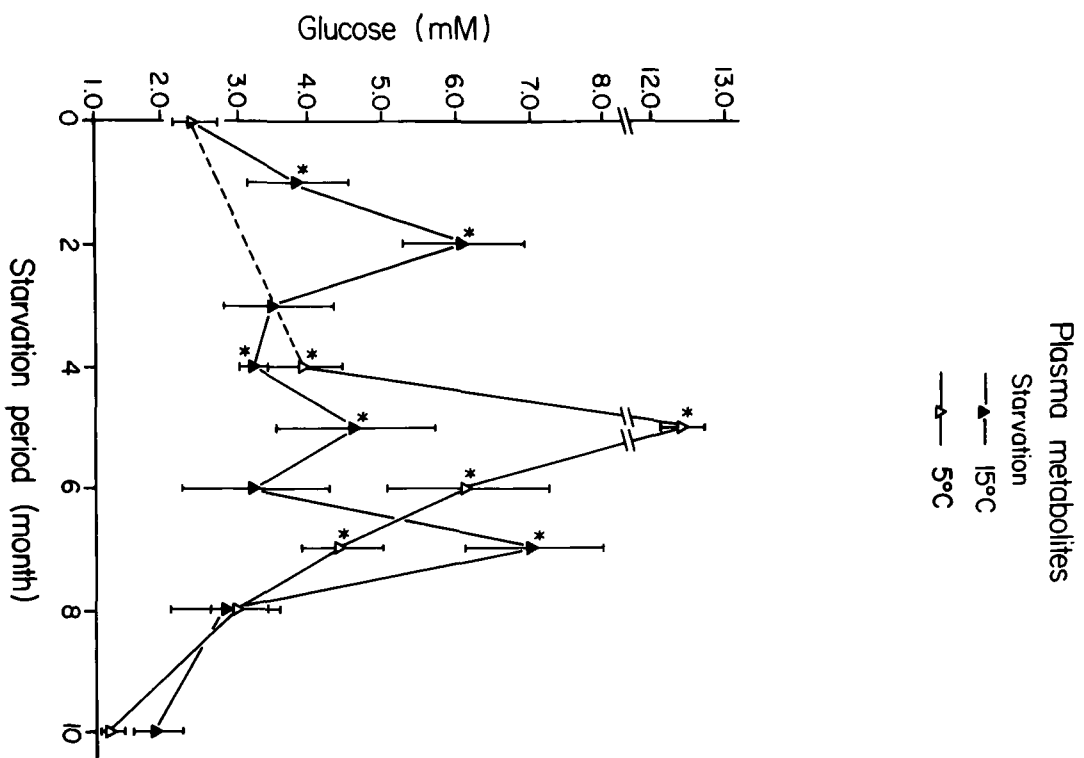
A) Plasma metabolites

Plasma glucose concentration in fed eels was approximately 2.4 mM (43.2 mg/100 ml). During the first seven months of starvation this value was significantly elevated at both temperatures (Fig. 1A). Two major peaks were observed at the 2nd and 7th months for the 15°C group, while the 5°C starved group showed only one peak at the 5th month. These elevated values decreased to normal at the 8th month and tended to be below control by the 10th month of starvation.

The concentration of plasma ninhydrin positive substances was significantly above control values for the first six months of starvation at both temperatures, with the 5°C group exceeding the 15°C

Figure 1. Effects of starvation period on eel plasma metabolites. St. Lawrence River eels were starved at $15 \pm 1^{\circ}\text{C}$ or at $5 \pm 1^{\circ}\text{C}$. Vertical bars represent the SEM of four eels during starvation and eight eels at time 0.

*Significantly different from time 0; t-test $P < 0.05$.



group during the 4th to 6th months (Fig. 1 C). These values returned to normal by the 7th month. Plasma protein concentrations (Fig. 1 B) were approximately 4.2 g per 100 ml in the fed eel, and only minor fluctuations were noted during starvation. The one major trend was that levels at 5°C were elevated above those at 15°C for most of the starvation period.

B) Liver metabolites

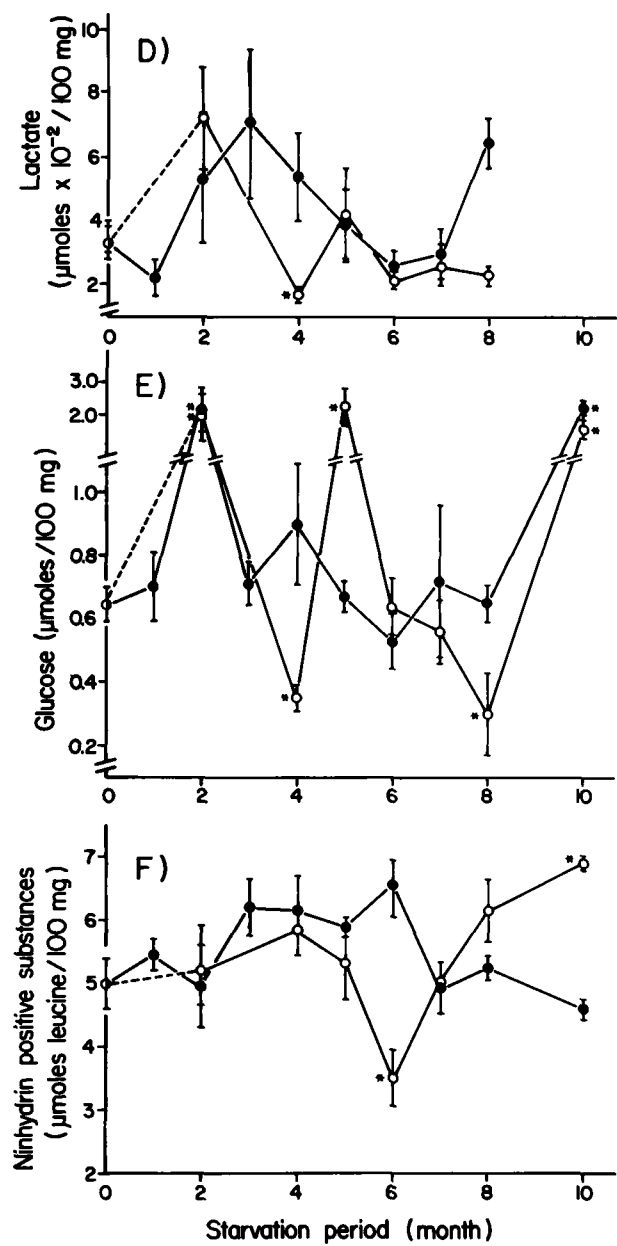
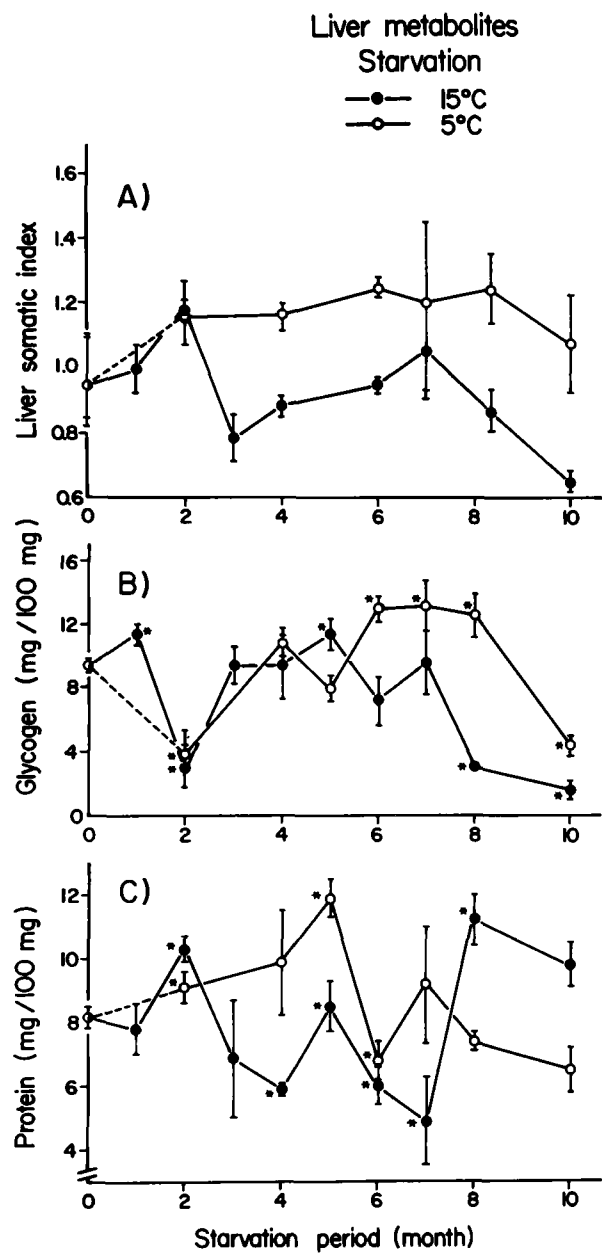
Liver somatic index increased slightly until the second month of starvation at 5°C and 15°C (Fig. 2 A). Thereafter, this index remained constant in eels starved at 5°C but it decreased in eels starved at 15°C. Significant differences were not observed at any time between control and starved eels, but were seen between the 5°C and 15°C starved groups.

An initial depletion of liver glycogen was observed at the 2nd month of starvation at both temperatures followed by a glycogen repletion at the 3rd month (Fig. 2 B). Thereafter, glycogen levels tended to be slightly above control until the 8th month where depletion again occurred. Liver protein increased significantly until the 5th month of starvation at 5°C after which it resumed control values (Fig. 2 C). During starvation at 15°C, eel liver protein concentrations tended to be lower than control except at the 2nd month, and post-seventh month.

Liver glucose concentrations was approximately 0.64 µmoles per 100 mg wet weight in fed eels, and major fluctuations in the value were noted during starvation at both temperatures (Fig. 2 E). The fluctuations tended to follow those of plasma glucose (Fig. 1 A), except the major increase in liver glucose was seen at the 10th month. The concentration of liver ninhydrin positive substances was approximately 5.0 µmoles per 100 mg wet weight in fed eels and no major changes

Figure 2. Metabolite profiles during starvation in eel liver. St. Lawrence River eels were starved at $15 \pm 1^{\circ}\text{C}$ or at $15 \pm 1^{\circ}\text{C}$. Vertical bars represent the SEM of four eels during starvation and eight eels at time 0.

*Significantly different from time 0; t-test $P < 0.05$.



occurred during starvation, except for the 5°C-group (Fig. 2 F). Plasma lactate values were estimated only in a few fish, since volumes were very small. Where available, a range of concentrations between 0.3 and 1.5 mM and 0.3 and 0.8 mM was noted during starvation at 15°C and 5°C, respectively. Liver lactate concentrations were not significantly different in control and starved eels during the 10 month experiment (Fig. 2 D).

C) White muscle metabolites

White skeletal muscle glycogen concentration was 15-times lower than the liver value but no significant change was observed during the 10 months of starvation at either temperature (Fig. 3 A). Protein concentration decreased significantly below control values by the 3rd month and tended to remain below control throughout, especially in the 15°C group (Fig. 3 B). There was appreciable protein recovery at 5°C, but the values still remained 20% below controls at the 10th month.

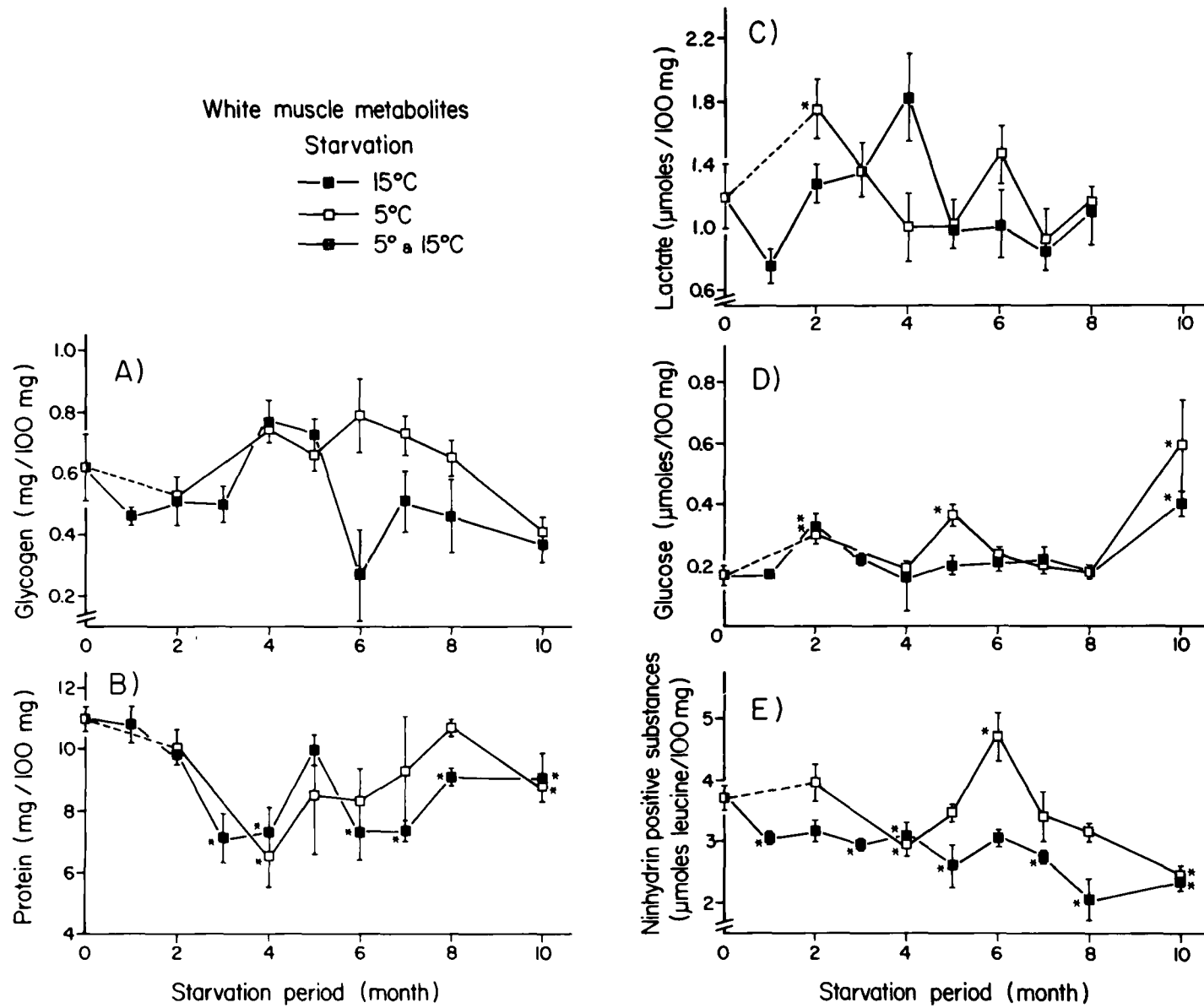
White skeletal muscle glucose concentration was 3.5-fold below the liver value in fed eels (Fig. 3 D). Some monthly variations were noted which correspond to those seen in plasma and liver, but these changes were of much lower absolute magnitude. Ninhydrin positive substances tend to decline during starvation at 15°C, while remaining relatively constant at 5°C (Fig. 3 E). White muscle lactate concentration was 36-times higher than the liver value, but no significant change was noted during the starvation period at either temperature.

II. EEL GLUCONEOGENESIS DURING STARVATION

Approximately 100 nmoles lactate per hr per 100 mg wet weight are incorporated into glucose under fed conditions (Fig. 4 A). This

Figure 3. Metabolite profiles during starvation in eel white skeletal muscle. St. Lawrence River eels were starved at $15 \pm 1^{\circ}\text{C}$ or at $5 \pm 1^{\circ}\text{C}$. Vertical bars represent the SEM of four eels during starvation and eight eels at time 0.

*Significantly different from time 0; t-test $P < 0.05$.



gluconeogenic rate increased tremendously during the first five months of starvation at 15°C (Fig. 4 A) and remained significantly above control for the entire 10 month period at 5°C, except at the 5th month (Fig. 5A). The highest rate was observed at the second month which corresponds in time with the first glycogen depletion in liver (Fig. 2 B) and the first high level of glucose in the plasma (Fig. 1 A) and tissues (Figs. 2 E, 3 D). Gluconeogenic rates from lactate were below fed control levels at the 8th and 10th months of starvation at 15°C.

Gluconeogenic rates from alanine were about half of those from lactate in the fed control eel (Fig. 4 B). These rates exceeded control values at the 2nd and 8th months of starvation at 15°C (Fig. 4 B) and the 2nd month at 5°C (Fig. 5 B); rates were equivalent (at 15°C) or below (at 5°C) control values at all other times. Only at the 8th and 10th months of starvation at 15°C did gluconeogenic rates from alanine exceed those from lactate (Fig. 4). Gluconeogenesis from aspartate was insignificant when compared to alanine and lactate, especially during starvation at 5°C (Figs. 4 C and 5 C), with the exception of months 2 through 4 where rates approximated those seen for alanine at 15°C.

The hormone glucagon is known to increase gluconeogenesis in the rat hepatocyte, and the principal mode of action is through cAMP. Therefore, glucagon and the soluble cAMP derivative dibutyryl cAMP were used in the eel hepatocyte preparation. These effectors were initially studied with 1 mM pyruvate and neither significantly increased gluconeogenesis (results not shown). Table 1 demonstrates that glucagon and dibutyryl-cAMP significantly stimulated lactate gluconeogenesis at the 8th and 10th months of starvation at 15°C. If there was an effect on amino acid gluconeogenesis, it was a trend towards

Figure 4. The distribution of radioactive substrates into eel hepatocyte metabolites. St. Lawrence River eels were starved at $15 \pm 1^\circ\text{C}$; livers were perfused 30 min with a 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4, and the isolated hepatocytes were incubated 1 hr at $15 \pm 1^\circ\text{C}$ in the standard Ca^{++} -phosphate buffer, pH 7.4, to which was added A) 1 mM lactate (1 μCi [$\text{U}-^{14}\text{C}$]-lactate) or a 1 mM amino acid mix (0.2 mM of each of the following amino acids: alanine, aspartate, glutamate, glycine and leucine) with B) 1 μCi [$\text{U}-^{14}\text{C}$]-alanine or C) 1 μCi [$\text{U}-^{14}\text{C}$]-aspartate. Vertical bars represent the SEM of two eels during starvation and 8 eels at time 0.

*Significantly different from time 0; t-test $P < 0.05$.

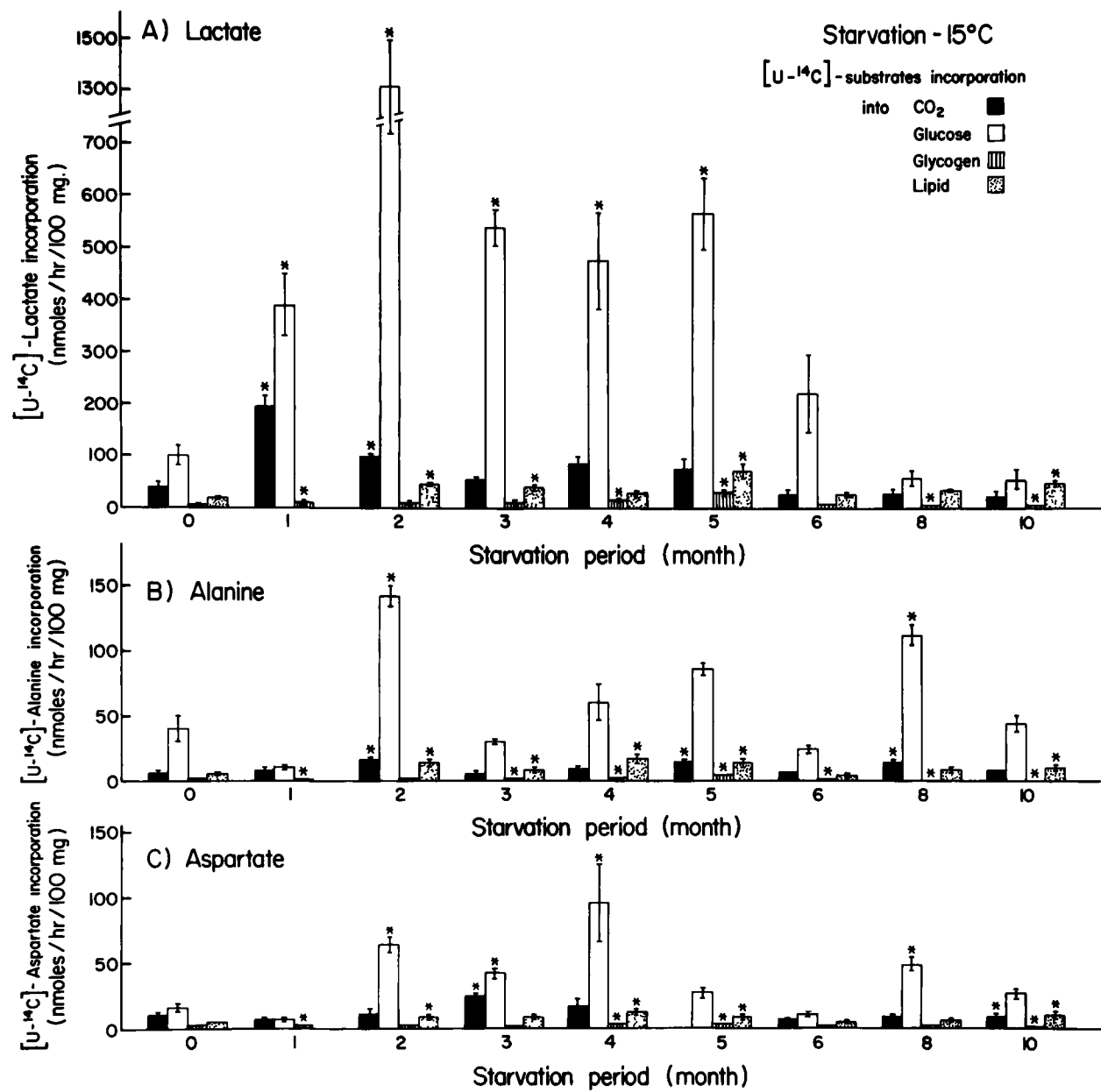


Figure 5. The distribution of radioactive substrates into eel hepatocyte metabolites. St. Lawrence River eels were starved at $5 \pm 1^{\circ}\text{C}$. All conditions are identical to those in Fig. 4, except that incubation temperature was $5 \pm 1^{\circ}\text{C}$.

*Significantly different from time 0; t-test $P < 0.05$.

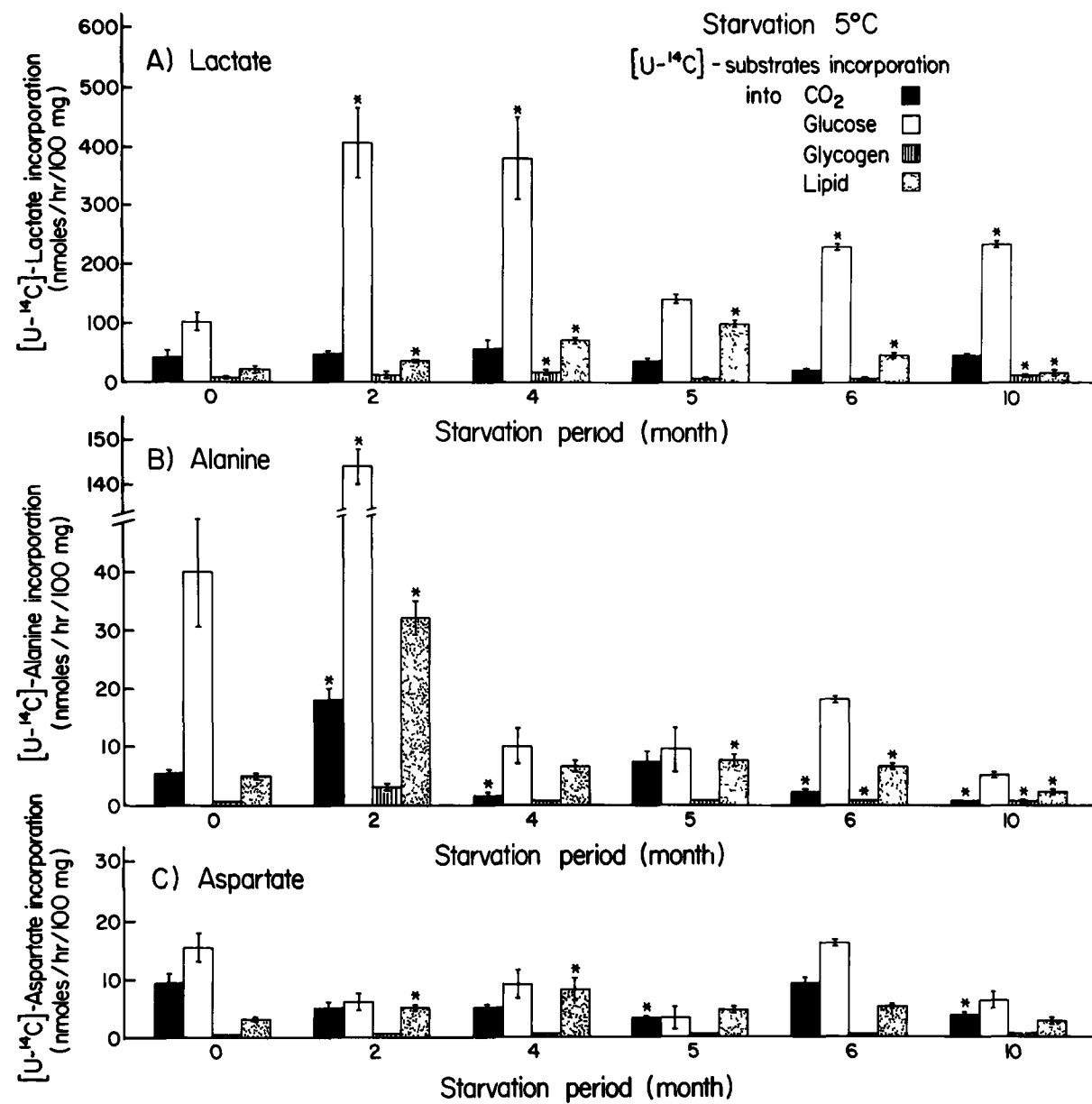


Table 1. Effects of glucagon (14.7 μ M) and dibutyryl cAMP (0.1 mM diB cAMP) on gluconeogenesis and glycogenesis in eel hepatocytes during late starvation at $15 \pm 1^{\circ}\text{C}$ and $5 \pm 1^{\circ}\text{C}$. Experimental details are listed in Fig. 4. Incubations of the hepatocytes were at the temperature of acclimation and in the presence of 1 μ Ci of radioactive substrates and cold substrates at the indicated concentrations. Results, from two eels, are expressed as MEAN $\pm$ SEM.

¹Hepatocytes were incubated in a 1 mM amino acid mixture (0.2 mM of the following amino acids: alanine, aspartate, glutamate, glycine and leucine).

*Significantly different from control; t-test $P < 0.05$.

a decrease with these effectors. No significant effects were seen at the 10th month of starvation for the 5 °C-group for either effectors on any substrate tested.

III. SUBSTRATE INCORPORATION (OTHER THAN GLUCOSE) DURING STARVATION

Although glucose and gluconeogenesis are the major fate of lactate, alanine and aspartate in the starved eel hepatocyte, other metabolic pathways were found to be significant.

As previously reported (Chaster 3), substrate incorporation into glycogen was very small (less than 2.5 nmoles/hr/100 mg), but a trend is discernible. From the 3rd to the 5th months of starvation at 15 °C, incorporation into glycogen from all substrates increased (Fig. 4), but by the 8th to 10th month these decreased to essentially zero. Glucagon and dibutyryl-cAMP decreased glycogenic rates only at the 8th month (Table 1). At 5 °C, rates of aspartate and alanine incorporation into glycogen remained relatively stable, whereas incorporation from lactate increased gradually to the 4th month (Fig. 5), and rates generally were above those found during late 15 °C starvation. No major effects of glucagon or dibutyryl-cAMP were noted at 5 °C (Table 1).

The rate of lactate oxidation in the fed control was approximately 50% of lactate incorporated into glucose (Figs. 4 A and 5 A). Except for the first two months, this rate did not differ significantly from the fed control during starvation at 15 °C or for the entire starvation period at 5 °C (Fig. 5 A). Oxidation of alanine and aspartate in the fed controls, were about 8- and 4-times lower than lactate, respectively (Fig. 4 B, C). No major trends were noted at either temperature with the exception of a general decline in oxidation rate as starvation

Table 2. Effects of glucagon (14.7 μ M) and dibutyryl cAMP (0.1 mM diBcAMP) on substrate incorporation into the CO₂ and lipid fractions of starved eel hepatocytes at $15 \pm 1^{\circ}$ C and $5 \pm 1^{\circ}$ C. Conditions are as in Table 1.

¹Hepatocytes were incubated with 1 mM amino acid mix (composed of 0.2 mM of the following amino acids: alanine, aspartate, glutamate, glycine and leucine)

*Significantly different from control, t-test $P < 0.05$.

Added Substrates	Substrate Incorporation into CO ₂			Substrate Incorporation into Lipid		
	(nmoles/hr/100 mg)			(nmoles/hr/100 mg)		
	Control	Glucagon (14.3 μ M)	diBcAMP (0.1 mM)	Control	Glucagon (14.3 μ M)	diBcAMP (0.1 mM)
a) <u>STARVATION 15°C</u>						
<u>8th month</u>						
-1.0 mM lactate	22.6 $\pm$ 9.1	26.6 $\pm$ 11.8	21.3 $\pm$ 8.7	29.3 $\pm$ 0.1	31.3 $\pm$ 3.5	30.2 $\pm$ 3.0
-0.2 mM alanine ¹	13.6 $\pm$ 0.8	12.2 $\pm$ 0.3*	11.6 $\pm$ 0.4*	6.7 $\pm$ 0.5	7.8 $\pm$ 0.5	7.3 $\pm$ 0.3
-0.2 mM aspartate ¹	7.1 $\pm$ 3.7	17.4 $\pm$ 6.1	17.2 $\pm$ 5.3	4.5 $\pm$ 0.5	4.1 $\pm$ 0.4	3.4 $\pm$ 0.4
<u>10th month</u>						
-1.0 mM lactate	21.5 $\pm$ 9.6	28.5 $\pm$ 17.9	25.9 $\pm$ 16.7	42.0 $\pm$ 3.8	42.8 $\pm$ 1.2	30.9 $\pm$ 8.8
-0.2 mM alanine ¹	8.1 $\pm$ 0.2	7.3 $\pm$ 0.4	6.3 $\pm$ 1.0	9.2 $\pm$ 1.0	9.4 $\pm$ 0.1	8.4 $\pm$ 0.7
-0.2 mM aspartate ¹	8.4 $\pm$ 0.1	10.5 $\pm$ 4.4	8.0 $\pm$ 1.6	8.4 $\pm$ 0.7	7.3 $\pm$ 0.3	7.1 $\pm$ 1.2
b) <u>STARVATION 5°C</u>						
<u>10th month</u>						
-1.0 mM lactate	43.5 $\pm$ 1.4	42.5 $\pm$ 2.0	43.2 $\pm$ 1.5	14.4 $\pm$ 1.1	13.3 $\pm$ 1.6	11.3 $\pm$ 0.4*
-0.2 mM alanine ¹	0.6 $\pm$ 0.1	0.6 $\pm$ 0.2	0.6 $\pm$ 0.1	1.9 $\pm$ 0.1	2.1 $\pm$ 0.1*	1.9 $\pm$ 0.1
-0.2 mM aspartate ¹	3.7 $\pm$ 0.2	3.4 $\pm$ 0.3	3.8 $\pm$ 0.3	2.2 $\pm$ 0.1	2.2 $\pm$ 0.1	1.9 $\pm$ 0.1*

progressed. Glucagon and dibutyryl cAMP had no effect at the 8th or 10th month of starvation at either temperature (Table 2).

As previously reported (Table III-7) substrate incorporation into the lipid fraction occurred only to the glycerol moiety, not the fatty acid chains. In general, all substrates at 15°C demonstrated a general increase in the incorporation into the lipid fraction up to about 5 months of starvation; thereafter, rates fell but remained significantly above the fed control values (Fig. 4). At 5°C, rates were generally higher during the early starvation period, but declined below values at 15°C by the 10th month (Fig. 5). This high rate during the first few months at 5°C may be associated with temperature adaptation, rather than starvation (see discussion). Variable effects of glucagon and dibutyryl-cAMP were seen with a generalized decline in most substrates noted (Table 2).

IV RATIOS

To compare the relative increase of gluconeogenic, glycogenic and lipogenic rates, three different metabolite ratios were calculated from the observed rates. If the ratios remain constant during starvation, the relative carbon flux along particular pathways must remain the same; however, a change in the ratio would indicate major disturbances in the competition between pathways for carbon.

A) Gluconeogenic: lipogenic ratio

Since all substrates were incorporated into glycerol not the free fatty acid chains of the lipid (Table III-7) this ratio represents the competition for dihydroxyacetone by aldolase for gluconeogenesis and αGPD for lipogenesis (Fig. I-2). In a fed eel, this ratio was

approximately 5.0 from all substrates used (Fig. 6). From lactate, ratios were significantly above the fed control for the first four months of starvation at 15°C followed by a gradual decline thereafter (Fig. 6 A). Ratios calculated from the two amino acid substrates did not differ significantly from the fed control except at the 2nd and 8th months which correspond to peak gluconeogenesis from the amino acids.

These ratios generally declined below the fed control at 5°C although at the 2nd and 10th months lactate values increased markedly (Fig. 6 B). This reduction in ratio again supports the importance of lipogenesis at cold temperatures.

B) Gluconeogenic: glycogenic ratio

This ratio represents the metabolic fate of glucose-6-phosphate between G-6-Pase for glucose release and glycogen synthetase for glycogen synthesis. In the fed eel, this ratio was between 50 and 120 depending upon substrate (Fig. 7). A simultaneous increase is noted in the ratio at the 2nd month of starvation at 15°C which corresponds to the low glycogen level in liver and high glucose level in the tissues (Fig. 7 A). From the 2nd to the 5th months, the ratio continuously decreased as incorporation into glycogen steadily increased (Fig. 4), followed by a gradual increase thereafter as glycogen levels decreased towards zero (Fig. 2B).

With the exception of aspartate at the 6th month ratios at 5°C did not differ from the fed controls.

C) Glycogenic: lipogenic ratio

This ratio represents the metabolic tendency of substrates towards glycogen and lipid. The fed control ratio varied between 0.06 and 0.13

Figure 6. Gluconeogenic: lipogenic ratio in the starved eel hepatocyte. Ratios were calculated from results of Figs. 4 and 5. Closed symbols, starvation at $15 \pm 1^{\circ}\text{C}$; open symbols, starvation at $5 \pm 1^{\circ}\text{C}$; ■ □ lactate; ● ○ alanine; ▲ △ aspartate.

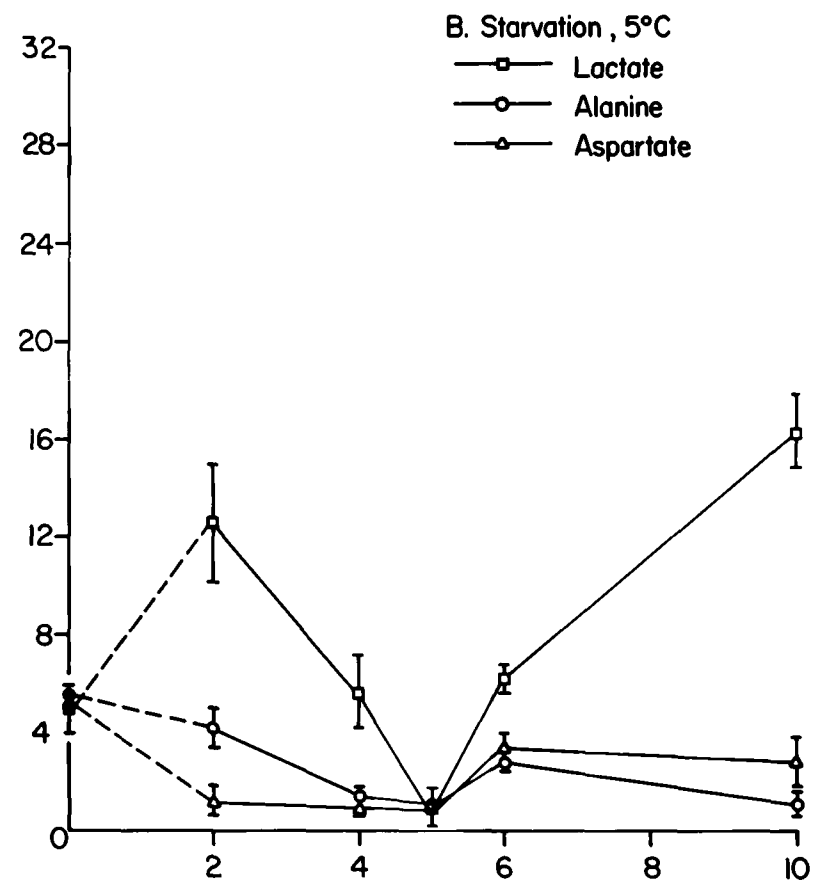
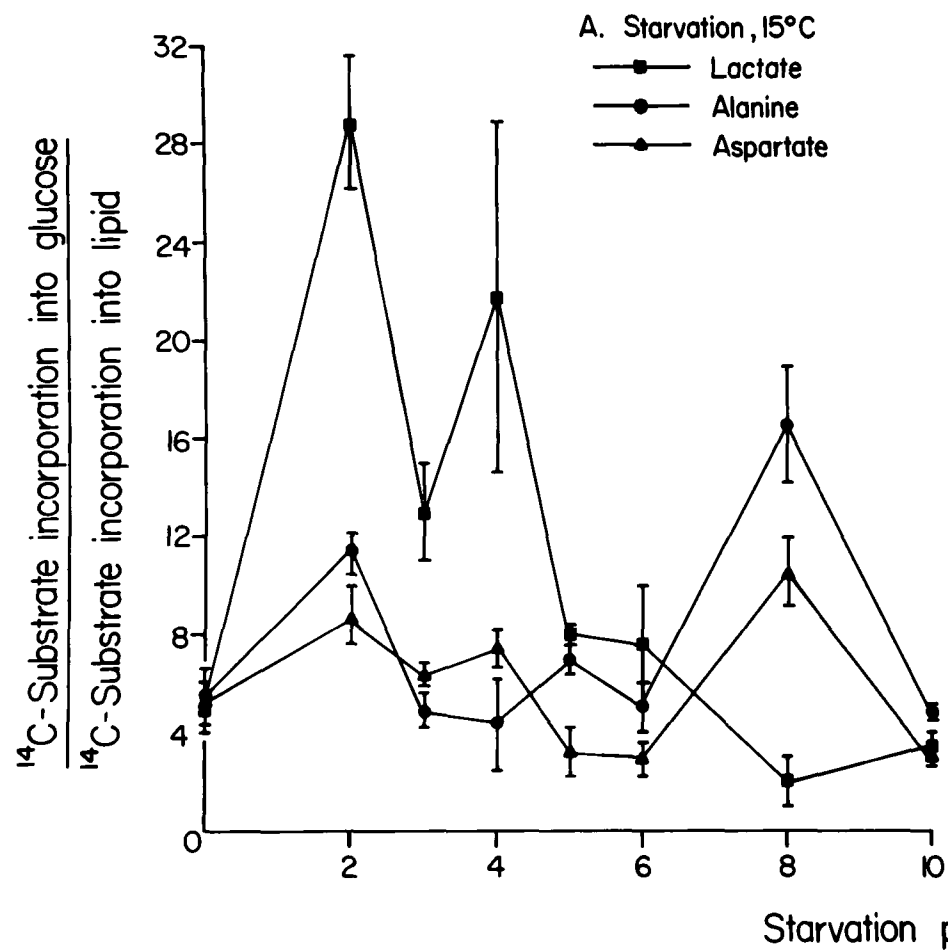
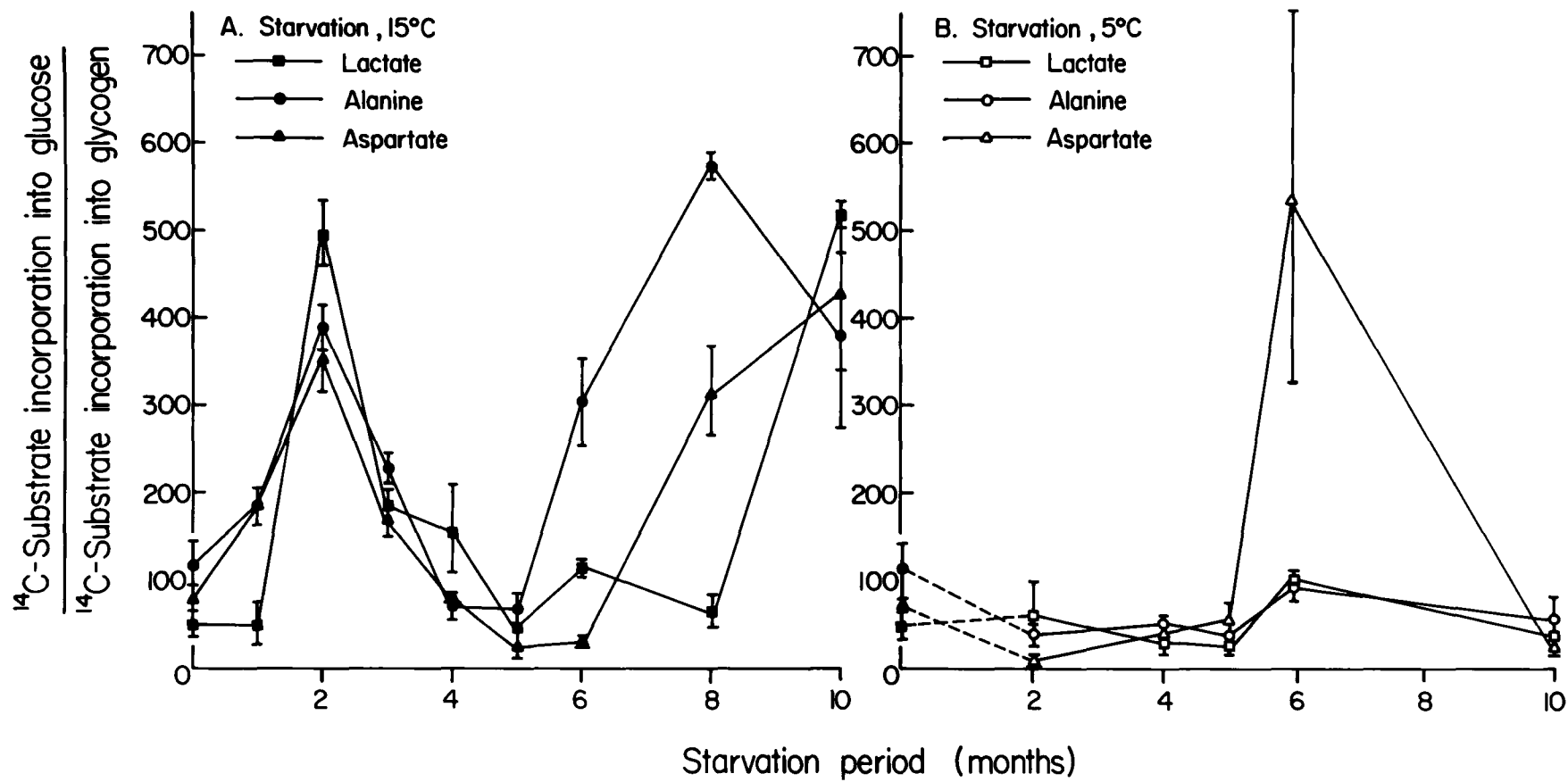


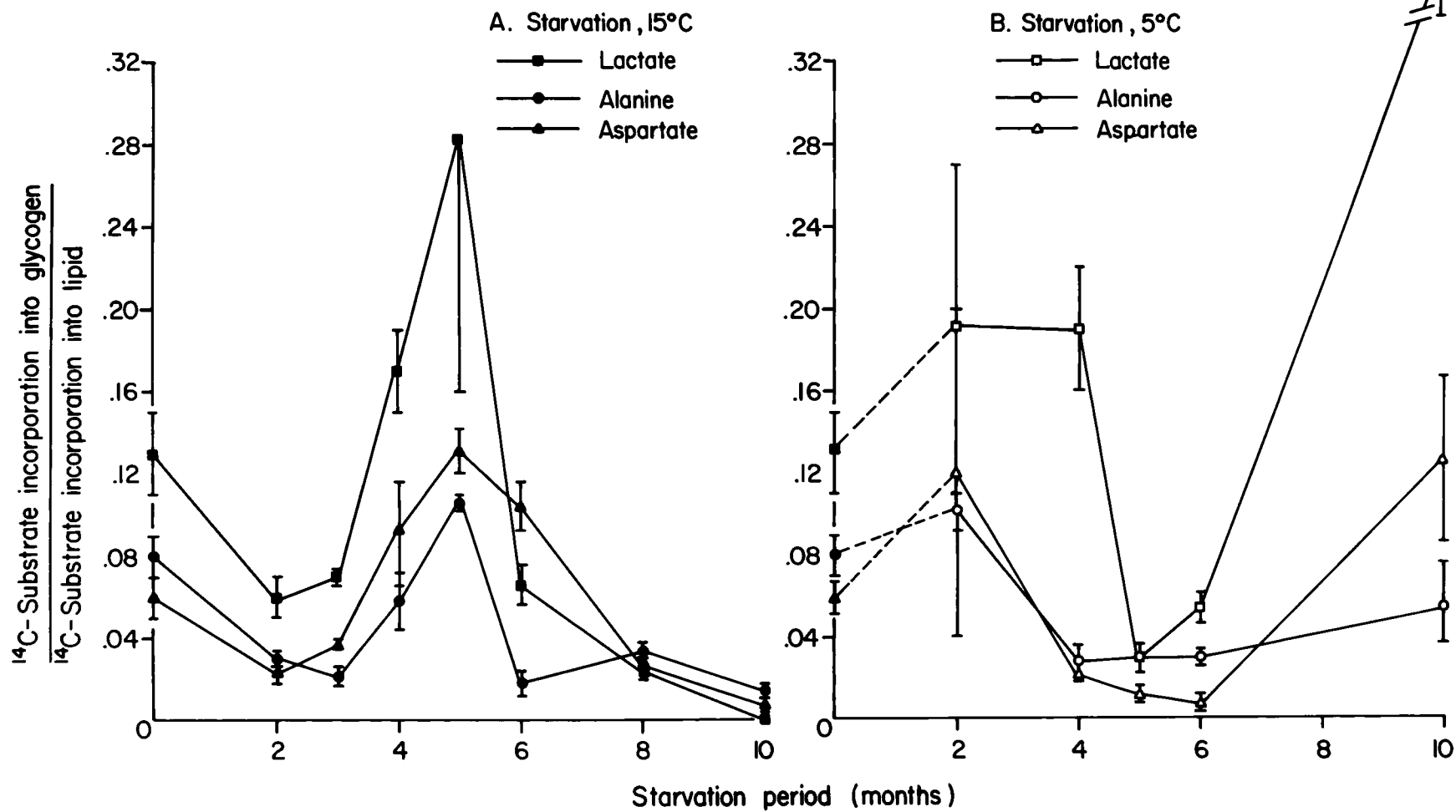
Figure 7. Gluconeogenic: Glycogenic ratio in the starved eel hepatocyte. Ratios were calculated from results of Figs. 4 and 5. Closed symbols, starvation at $15 \pm 1^{\circ}\text{C}$; open symbols, starvation at $15 \pm 1^{\circ}\text{C}$; ■ □ lactate; ● ○ alanine; ▲ △ aspartate.



depending upon substrate (Fig. 8). This low ratio in the fed eel hepatocyte demonstrates the importance of lipid as a storage fuel compared to glycogen. An initial decrease was observed at 15°C until the 2nd month followed by a significant increase between the 3rd and 5th months of starvation (Fig. 8 A). This increase corresponded with the increase of substrate incorporation into glycogen (Fig. 4). After the 5th month, ratios decreased towards zero as substrate incorporation into glycogen tended towards zero (Fig. 4). At 5°C, ratios remained constant for the first four months before a decrease occurred at the 5th month of starvation (Fig. 8 B). At the 10th month, ratios with lactate and aspartate were significantly above the fed control.

Figure 8. Glycogenic:lipogenic ratio in the starved eel hepatocyte.

Ratios were calculated from results of Figs. 4 and 5. Closed symbols, starvation at $15 \pm 1^{\circ}\text{C}$; open symbols, starvation at $5 \pm 1^{\circ}\text{C}$; ■ □ lactate; ● ○ alanine; ▲ △ aspartate.



DISCUSSION

Plasma glucose levels were maintained significantly above the fed control during seven months of starvation in the American eel at both acclimation temperatures (Fig. 1). In the European eel, no trend in plasma glucose concentration was observed during starvation probably because the time between sampling periods was too large (Larsson and Lewander, 1973; Dave et al., 1975). Moreover, Larsson and Lewander (1973) showed that plasma glucose concentrations after 96 days of starvation at 9 °C are significantly higher than fed control values, whereas Dave et al. (1975) showed a reverse situation. In the Northern pike, blood glucose concentration was below the fed control only after 3 months of starvation at 10 °C (Ince and Thorpe, 1976a) and no difference was observed in trout starved one month or fed a high protein diet (Cowey et al., 1977a). O'Boyle and Beamish (1977) showed that both serum glucose and muscle glycogen rise during winter starvation probably as a result of decreased liver oxidation of glucose.

When blood glucose values increased, such as the 2nd month, the glucose concentrations in liver and muscle increase concurrently. Since glucose transported into rat liver and muscle is nearly at equilibrium with plasma glucose concentration (Baur and Heldt, 1975; Newsholme and Start, 1976), such changes would be expected assuming eel tissue glucose transport is also near equilibrium. Interestingly, the free glucose concentration found in the eel liver is similar to that reported for rat liver (Shimassek et al., 1973).

The most important question to be asked concerns the maintenance of this high blood glucose concentration. There are at least three possibilities: 1) eel activities decrease during starvation which

ultimately decreases glucose demand; 2) rates of glycogenolysis and/or 3) gluconeogenesis increase and glucose production exceeds glucose utilization. It is evident that during 10 months of starvation any or all of these possibilities may be important at some period.

I. EFFECTS OF STARVATION ON FISH ACTIVITIES

Starvation in fish is associated with a decrease in activity, preserving nutrient stores for maintenance functions (Beamish, 1964) showed that rates of oxygen uptake by trout and white sucker decrease significantly during the first five days of starvation at 10°C and then remain constant for at least 25 days. Rates of CO₂ production from [6-¹⁴C]-glucose and [1-¹⁴C]-glucose in 5 month starved carp are half of those observed in fed carp (Creach and Murat, 1974). It is well known that between 5-10°C, European eels are inactive and completely hidden in the mud (Nyman, 1972; Sinha and Jones, 1975). Although the eel used in this study did not have access to mud, their activity was observably reduced compared to the 15°C fed or starved animals. Therefore, the 5°C starved group would represent a "natural" stress, but at 15°C, starvation is unnatural and activities may not decrease.

Mayerle and Butler (1971) proposed that American eels starved at 5°C are less active than those starved at 15°C based on two experimental observations. First, plasma glucose concentrations were higher in eels starved 5 months at 5°C compared to those starved at 15°C. A similar result was found in this study (Fig. 1), but a similar conclusion should not be drawn since it represents the only period of starvation where eels starved at 5°C had a plasma glucose value which was significantly higher than those starved at 15°C.

The second observation was a higher plasma lactate value in eels starved at 15°C than those starved at 5°C. Unfortunately, plasma lactate values could not be consistently estimated in this study, although a similar trend was observed. White muscle lactate values did not differ significantly between eel starved at 5°C and 15°C (Fig. 3), but this may result from a lower diffusion rate at low body temperature (Black et al., 1961) rather than a similar production rate at both temperatures. A difference in diffusion rates is suggested by the smaller range of values at 5°C than the range at 15°C. Larsson and Lewander (1973) also report slightly increased plasma lactate values after 95 and 145 days of starvation in the European eel.

Thus the possibility that a decrease in muscular activity accounts for the rise in plasma glucose cannot be excluded, but it does not seem to be the major explanation.

II. RATES OF GLYCOGENEOLYSIS DURING STARVATION

Since low glycogen quantities were found in white skeletal muscle and no significant decrease was observed during starvation (Figure 3), glycogenolysis in white skeletal muscle does not appear to be an important energy source during starvation. Low muscle glycogen levels have also been observed in Northern pike (Ince and Thorpe, 1976a) and European eel (Larsson and Lewander, 1973) which is quite unlike mammals (Hermansen and Vaage, 1977). No explanation has been put forward for this observation although it would suggest a minor role for glycogen storage in many fish.

Liver glycogenolysis may be an important source of plasma glucose, but only at the 2nd month of starvation because it is the only period

where liver glycogen depletion is correlated with high plasma and tissue glucose (compare Figs. 1 and 2). Thereafter, glycogenesis exceeds glycogenolysis since glycogen stores were replenished and remained sometimes above fed control until the 8th month. Significant depletion occurred by the 10th month (Fig. 2), but blood glucose values were below fed control values at that time (Fig. 1).

Compared to the European eel, significant depletion of liver glycogen was observed only after 145 days of starvation at 5 °C, but not after 95 days (Larsson and Lewander, 1973; Dave et al., 1975). These differences may be the result of some habitat or life history differences between the two eel species (Vladykov, 1955). Although liver lipid and protein mobilization in the carp during starvation precede that of liver glycogen Creach and Serfaty (1974) showed that liver glycogen is significantly reduced during starvation. Similar patterns were observed in three months starved Northern pike (Ince and Thorpe, 1976a) and in rainbow trout (Cowey et al., 1977a). Cowey et al., (1977b) have suggested, however, that glycogen is not an immediate source of glucose since there is a very low net flux between glycogen and glucose.

In both the American and European eels, liver glycogenolysis may represent an important source of blood glucose, but probably for a very short period during such an extensive starvation. Therefore, as suggested by Cowey et al. (1977b), gluconeogenesis appears as the major source of blood glucose during most of the starvation period, a result which is consistent with the mammalian literature (Owen et al., 1969; Walter, 1973).

III. GLUCONEOGENESIS DURING STARVATION

If gluconeogenesis represents an important source of blood glucose during starvation in the American eel, an increase in gluconeogenic capacity would be predicted in those organs participating in this activity. This study and the previous one (Chapter 3) deal with the eel liver, but Savina and Woztczak (1977) have reported that lamprey muscle is capable of glycerol gluconeogenesis. Therefore, increases in gluconeogenic rates in the liver, coupled to an availability of suitable substrates would support gluconeogenesis being an important component in the starvation strategy of the eel.

A) Gluconeogenic rates during starvation

Lactate appears to be the predominant substrate for gluconeogenesis during starvation based upon three experimental findings: 1) gluconeogenic rates increased from lactate, but generally not from amino acids during starvation (Figs. 4, 5); 2) lactate gluconeogenesis was as high as 50-70 times that of alanine or aspartate gluconeogenesis; and, 3) glucagon and dibutyryl cAMP increased gluconeogenesis only from lactate in fed (Chapter 2) and late starved (Table 1) eel hepatocytes.

During the 2nd month of starvation at 5°C and 15°C, however, alanine gluconeogenesis was significantly above the fed control (Figs. 4 and 5) while liver glycogen stores were severely depleted (Fig. 2). This situation reflects the mammalian system where gluconeogenic rates from amino acids, and especially alanine, increase after total liver glycogen depletion (Cahill, 1976). A similar increase in both alanine and aspartate gluconeogenesis occurred at the 8th and 10th month at 15°C; again this is related to low liver glycogen values. Thus, amino acid gluconeogenesis may be important in the eel when carbohydrate

stores are depleted below some critical level, but this would depend upon substrate availability (see below).

In the 24 hr starved rat, alanine, glycine, aspartate, glucamate and leucine concentrations in the portal vein approximate 266, 253, 14, 64 and 110 μ M, respectively (Aikawa et al., 1973). In their study on amino acid gluconeogenesis, Mallette et al., (1969) have used similar concentrations of amino acids to estimate physiological rates of gluconeogenesis. Amino acid concentrations in the American eel blood have not yet been measured but in order to approximate "in vivo" conditions an amino acid mixture had also been used, except that the concentrations of the 5 amino acids were all at 0.2 mM. The 1 mM lactate concentration falls in the range observed during starvation; i.e., 0.3 to 1.5 mM. Consequently, the rate of gluconeogenesis reported in this study should approximate an in vivo situation. The only possible exception could be the 2nd month of starvation since high concentrations of ninhydrin-positive substances were estimated in the caudal vein, but it was already proposed that amino acid gluconeogenesis at that month was important.

Major differences in total gluconeogenic flux can be noted between the 5°C and 15°C groups. Over the first 5 months at 15°C, total flux exceeds that at 5°C by at least 30%. Since blood glucose levels are essentially identical between these two groups, this would be supportive of an enhanced activity in the 15°C group. The 6th through the 10th month is associated with a decline in flux below that of the fed control. This occurs only in the 15°C group not the 5°C group.

This apparent decrease in eel hepatocyte gluconeogenic capacity at 15°C was probably a result of a general decrease in gluconeogenic enzyme activities, but not a decrease in enzyme concentrations. The fact that both glucagon and dibutyryl cAMP activated lactate gluconeogenesis at 15°C more than at 5°C, suggests that the enzymes were present but their activities were suppressed. Thus at 15°C the exhaustion of some critical liver metabolite(s) or some circulating substance(s) (eg., hormones) may be responsible for the observed decrease in gluconeogenesis. At 5°C, these conditions did not exist, so activities were always maximal and therefore insensitive to hormone or metabolite activation.

Thus, starvation in the American eel is associated with increased gluconeogenic rates principally from lactate. Amino acids act as gluconeogenic substrates principally when liver glycogen levels are depleted below some critical point. Major changes also occur during late starvation in both acclimated eel groups which probably relate directly to changes in liver metabolites or plasma factors.

B) Source of gluconeogenic substrates during starvation.

If these observed increases in gluconeogenic rates are physiologically important during starvation in the eel, the appropriate substrates must have been present. The absence of significant decreases in white skeletal muscle lactate during starvation (Fig. 3) indicates that lactate was still available for liver gluconeogenesis.

In a comparative study, Phillips and Hird (1977) report relatively high levels of muscle lactate in the Australian eel compared to rats, but lower plasma lactate values. They suggested that lactate could serve as an important gluconeogenic precursor for this reason, and

in fact found very high lactate gluconeogenesis. Cowey et al. (1977a) predicted that lactate is of less quantitative importance as a gluconeogenic substrate in the carnivorous trout (and fish in general) than in the omnivorous mammal. Recently, a large body of literature is appearing (see review of Bennet, 1978) which indicates that lower vertebrates, including fish, rely more heavily on anaerobic (i.e., lactic acid production) than aerobic metabolism. Although these data are equivocal, it is premature to eliminate lactate as an important gluconeogenic substrate, especially when rates are so large. As discussed in Chapter 1, lactate gluconeogenesis only recycles glucose and will not result in net glucose synthesis. Thus, other non-carbohydrate substrates are required to obtain net glucose synthesis.

One possible substrate source is amino acids release from muscle proteolysis as found in mammals (Waterlow, 1968; Felig and Wharen, 1974; Cahill, 1976). Significant depletion of proteins in white skeletal muscle was observed at the 3rd and 4th months of starvation at 5 °C and 15 °C. This period just followed the highest gluconeogenic rates observed from all substrates at the preceding month, especially at 15 °C. A similar situation was observed at the 8th month of starvation at 15 °C (compared Figs. 3 and 4). These results suggest an increase in white skeletal muscle proteolysis and an increase in amino acid flux from white skeletal muscle to liver for gluconeogenesis. An increased amino acid flux is indicated since the concentration of ninyhydrin positive substances in white skeletal muscle were significantly below while those in plasma were significantly above fed control values. The apparent lack of significant proteolysis in white skeletal muscle at the 2nd month may be explained by the fact that skeletal muscle tissue

constitutes a major tissue mass and, therefore, a lag period might occur before any demonstrable protein depletion is detected.

Johnston and Goldspink (1973) reported a 83% decline in white muscle mass in the plaice starved a total of 14 weeks. Also, Cowey et al. (1977a) found that injected alanine is rapidly incorporated into glucose with a peak incorporation occurring 6 hrs after injection. Both studies suggest amino acid release is potentially an important substrate source for liver gluconeogenesis.

The results presented here differ from mammals in three important aspects. First, amino acid gluconeogenesis increases only for short time periods during an extensive starvation period; second, white skeletal muscle protein levels gradually increases back towards initial values during the last 6 months of starvation (Fig. 3); and third, eel liver protein content decreases (Fig. 2), while rat liver protein tends to increase during starvation (Eisenstein, 1967; Waterlow, 1968; Cahill, 1976). Since the liver is the main site of plasma protein synthesis in eel (Kenyon, 1967) and rat (Dick and Gluid, 1976), but since plasma protein levels did not decrease during starvation (Fig. 1), liver protein synthesis is not necessarily impaired. This result could suggest that eel protein could play an important precursor role in liver gluconeogenesis, but further studies are needed to verify this hypothesis.

Thus, during starvation white skeletal muscle proteolysis may occur to provide amino acids for liver gluconeogenesis. But, a general protein loss in other tissues, such as the liver, and the relatively low gluconeogenic rates from amino acids compared to lactate may suggest other roles for amino acids during starvation, such as a direct

amino acid oxidation for energy production. The eel hepatocyte, under the conditions of these experiments, will utilize lactate, presumably derived as an end product of tissue metabolism, efficiently as a gluconeogenic substrate.

C) Gluconeogenic controls during starvation

Hayashi and Ooshiro (1975) reported that both epinephrine and cAMP (the product of glucagon activation), stimulate lactate gluconeogenesis in the perfused Japanese eel liver. Alanine gluconeogenesis was stimulated by injected glucagon in toad fish (Tashima and Cahill, 1964), but these effects depend upon the source of glucagon and the fish species (Epple, 1969).

Under our experimental conditions, employing both the fed (Chapter 3) and late starved (Table 1) eel hepatocyte, glucagon and dibutyryl cAMP significantly increased only lactate gluconeogenesis, but not lactate and amino acid gluconeogenesis as reported in the rat preparation (Ross et al., 1967a, b). This stimulation could have been achieved by increasing pyruvate utilization by the gluconeogenic pathway as in mammals (Eisenstein, 1967; Elliot and Pogson; 1977). Murat et al. (1978) reported that the specific PEP CK inhibitor, 3-mercaptopycolinic acid, prevents glucagon-induced hyperglycemia in carp which is strong support for a similar action of glucagon in both mammals and fish. This would, however, demand some control over the amino acid transferases, especially alanine transaminase to prevent an increase in amino acid gluconeogenesis. Thus, control of gluconeogenesis in the eel hepatocyte is not equivalent to that reported in mammals.

Cortisol has been demonstrated to stimulate amino acid release, especially alanine, by skeletal muscle (Karl et al., 1976), and to

increase both liver gluconeogenesis and glycogenesis, especially from amino acids (Kendysh and Moroz, 1972) by increasing the activity and synthesis of all key gluconeogenic enzymes and amino acid transaminases (Eisenstein, 1967). A possible link between corticosteroid regulation of protein catabolism and gluconeogenesis in order to maintain carbohydrate levels in fish has been proposed by Woodhead (1975). Cortisol is responsible for the maintenance of eel liver and muscle glycogen and blood glucose levels (Butler, 1968) and increases lactate gluconeogenesis in the eel hepatocyte preparation (Chapter 3). The cortisol effect in eels, where it has been studied, appears similar to those reported in mammals.

Data collected in this study could be explained by the action of these two hormones, glucagon and cortisol. Since both starvation and glucagon only stimulate lactate gluconeogenesis, not alanine or aspartate gluconeogenesis, it may be suggested that glucagon has an important role in blood glucose homeostasis throughout the entire starvation period. Woodhead (1975), based on extensive literature, have proposed a corticosteroid regulation between protein catabolism and gluconeogenesis during starvation to maintain carbohydrate level constant. Results, from this study, suggest that amino acids play a minor role as a source of gluconeogenic substrates. During the 2nd month of starvation at both 5°C and 15°C and the 8th and 10th month at 15°C, amino acid gluconeogenesis becomes relatively important. During these periods, therefore, an enhanced plasma cortisol would be expected. A preliminary report by Huta (this laboratory, unpublished) does suggest enhanced transaminase activities in eel liver under 1-2 months and long term starvations. Similar observations have been reported in mammals under cortisol injection (Eisenstein, 1967).

Thus, the gluconeogenic rates during starvation in the rat and eel livers are probably adaptations to different degrees of starvation. If gluconeogenic rates from amino acids in eel liver were similar to those reported for rat liver, the amount of protein catabolism required over a 6 month starvation period would severely interfere with locomotor activities since few amino acids are efficient substrates. If an eel, starving at 5°C, reduces its activities and, thus reduces its glucose demand, glucose recycling via lactate gluconeogenesis may be sufficient to maintain blood glucose under glucagon stimulation if the oxidation of other metabolites, such as triglycerides, increases. The large increase in amino acid gluconeogenesis at the 2nd month is probably associated with an adjustment to long term starvation, through cortisol, while that observed at the 10th month at 15°C is associated with an unnatural, stressful starvation, when storage metabolites have dropped below critical levels and muscle protein is the only remaining energy sink.

IV. EFFECTS OF STARVATION ON OTHER METABOLITE INTERCONVERSIONS

In the carp, a winter starvation largely suppresses [6-¹⁴C]-glucose incorporation into glycogen, but has no effect on its incorporation into lipid (Creach and Murat, 1974). O'Boyle and Beamish (1977) report that during the winter starvation of the lamprey, Petromyzon marinus L., both PK/FDPase and HMSD*/FDPase ratios decrease tremendously in liver and

* HMSD, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are assayed together and referred to as the hexose monophosphate shunt dehydrogenases (HMSD).

muscle indicating an increase in glucose incorporation into fat and a relative increase of lipogenesis over gluconeogenesis.

In the fed and starved eel hepatocyte, glucose production from the three substrates predominates over oxidation, glycogenesis and lipogenesis, but throughout starvation the relative carbon flux towards these products differ. In the fed eel hepatocyte, the label is incorporated only into the glycerol component of the lipid (Chapter 3) and it is assumed that the pattern is unaffected by starvation since no activity of citrate cleavage enzyme and malic enzyme was detected after three months of starvation at 15°C (Aster, unpublished results). In this study, the source of the fatty acids necessary for the esterification of glycerol remains unknown, but Shrago et al. (1978) have reported that in fed isolated rat adipocyte, although [14 C]-pyruvate and [14 C] lactate are incorporated in both the fatty acid and glycerol fractions of lipid, the amount of glycerol produced and incorporated exceeded the amount of fatty acids produced and incorporated into lipid. Thus, in both the eel hepatocyte and rat adipocyte, incorporation of newly synthesized glycerol into lipid does not depend entirely on newly synthesized fatty acid.

In a 48 hr starved rat adipocyte, the difference between glycerol and fatty acid produced and incorporated into lipid is enhanced, but the total amount of glycerol produced and incorporated into lipid is 10-times lower than in the fed rat adipocyte. Also, fatty acid synthesis is almost nil in starved rat adipocytes (Shrago et al., 1978). These decreases in glycerol esterification and fatty acid synthesis and a concomitant increase in gluconeogenesis (Ross et al., 1967) in the rat during starvation is in agreement with the reciprocal relationship between gluconeogenesis and lipogenesis (Nikkila, 1973). This

relationship does not hold in the eel system.

During starvation at 15°C both gluconeogenesis and glycerol esterification increased in eel hepatocytes until the 5-6th month followed by a decrease thereafter and, thus the gluconeogenic: lipogenic ratio remained relatively constant from amino acids up until the last months of starvation while that for lactate initially increased followed by a gradual decline (Fig. 8A). This is evidence for the absence of a reciprocal relationship between gluconeogenesis and lipogenesis as reported for the rat. During starvation at 5°C, both gluconeogenesis and glycerol esterification also increased, but the gluconeogenic: lipogenic ratio tended to decrease especially during the early part of starvation (Fig. 8A). This may have been a result of thermal adaptation (see Hochachka and Somero, 1972).

Glycogen production does not represent a predominant product of eel hepatocyte metabolism which is consistent with reports in the rat hepatocyte (Walli, 1977). Only between the 3rd and 5th months of starvation at 15°C was there any appreciable change in glycogenesis (Fig. 7A), and it was at that time when liver glycogen levels were replenished (Fig. 2). It was also at this time when lipogenic rates changed relative to glycogenesis, while at all other times, lipogenic flux exceeded glycogenesis by approximately an order of magnitude (Fig. 8).

Two important questions arise from these data: 1) which substrates are oxidized for the production of energy (ATP, GTP) and reducing equivalents (NADH, NADPH) needed to sustain the increased liver anabolic activity; and 2) is the observed increase in gluconeogenic rates due to a higher glucose demand or a generally higher anabolic capacity of the eel liver to provide adequate metabolites during food deprivation?

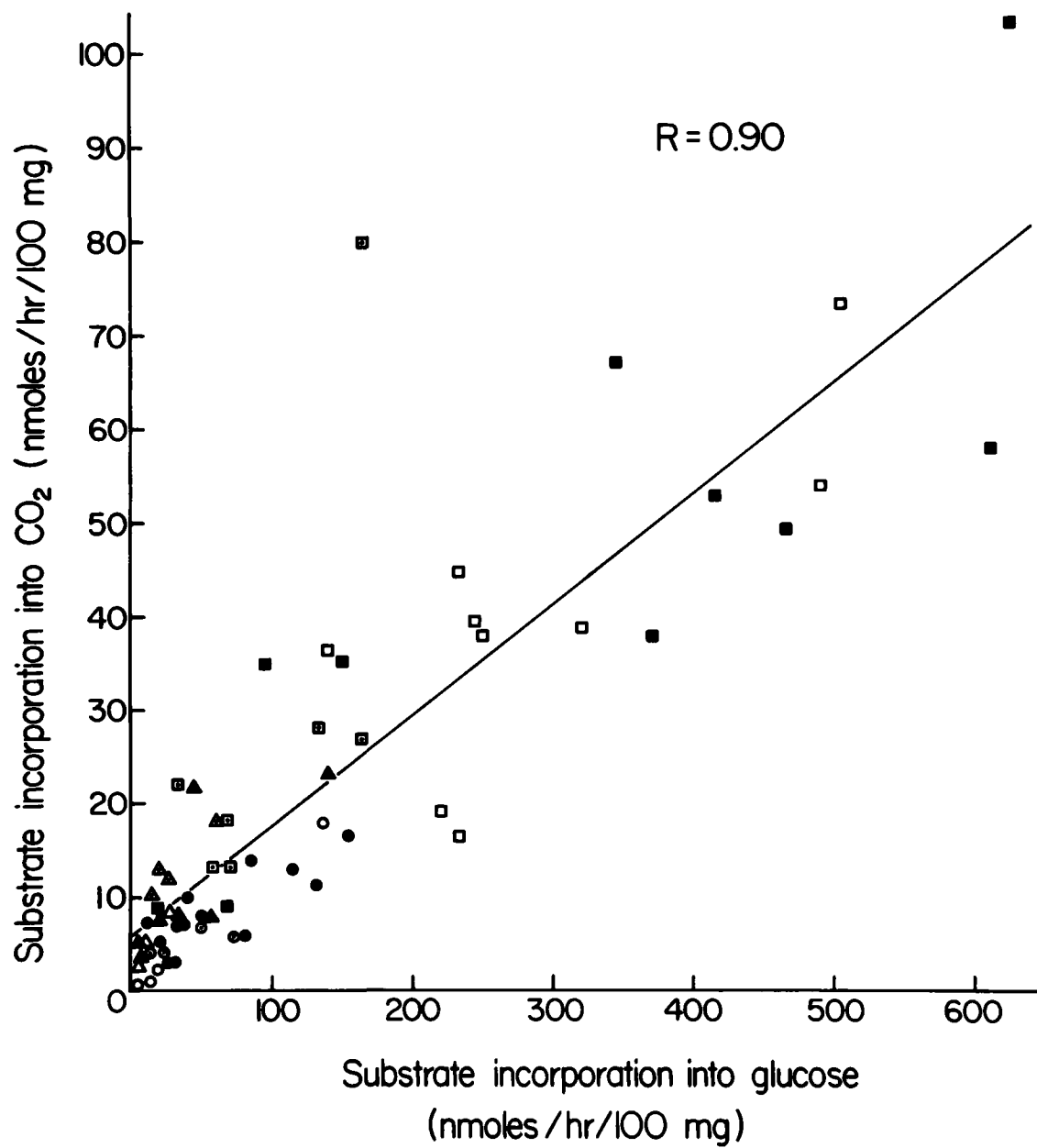
Initially, it is apparent that a direct correlation exists between substrate incorporation into glucose and CO_2 (Fig. 9) in both fed and starved eels. The slope of this line, 0.123 moles of substrate incorporated into glucose approximates the value of 0.133 if it is considered that the oxidation of 2 moles of pyruvate provides the 30 moles of ATP required for the incorporation of 15 moles of pyruvate into glucose. Thus, energy and reducing equivalents necessary for anabolic activities were derived from the substrates used for gluconeogenesis, not from lipolysis as has been established in mammals (Stucki et al., 1972).

The second question is more difficult to answer. At 5°C , an increase in both gluconeogenesis and lipogenesis occurred over the first 5 months from lactate and alanine in particular (Figs. 5, 6B, 7B and 8B). Similarly, glycogenesis from lactate increases at 5°C . The picture at 15°C is more complex probably as a result of the high gluconeogenic rates observed over the 2nd to 5th months (Figs. 4, 6A, 7A and 8A). Under "natural" starvation conditions at 5°C , a general anabolic increase is suggested while at 15°C , which is a more stressful starvation gluconeogenesis is preferentially activated.

V. THE 5th MONTH OF STARVATION

A general decrease in substrate incorporation into CO_2 , glucose, glycogen and lipid was observed around the 5th and 6th months of starvation at 5°C . This period corresponds in time to the end of a natural winter fast where water temperatures begin to rise and fish have access to food. When starvation was extended beyond this time at 5°C , eels were able to support gluconeogenic rates and liver glycogen values above

Figure 9. Correlation between substrate incorporation into CO_2 and into glucose. These points are taken from the individual values shown in Figs. II-5, II-7, III-4 and III-5. $\square \odot \Delta$ fed eel hepatocytes; $\blacksquare \bullet \blacktriangle$ starved (15°C) eel hepatocytes; $\square \circ \Delta$ starved (5°C) eel hepatocytes; $\blacksquare \blacksquare \square$ $1 \mu\text{Ci}$ $[\text{U}-^{14}\text{C}]$ -lactate; $\odot \bullet \circ$ $1 \mu\text{Ci}$ $[\text{U}-^{14}\text{C}]$ -alanine; $\blacktriangle \triangle \triangle$ $1 \mu\text{Ci}$ $[\text{U}-^{14}\text{C}]$ -aspartate.



fed control suggesting a fail-safe mechanism is available to the fish until better conditions of temperature and food occur. Such a mechanism is not available in the 15^o C-acclimated eels since gluconeogenic capacities were reduced and liver glycogen stores were significantly depleted. The control mechanisms associated with these changes remain unknown but they could be part of a biological cycle (rhythm) since these changes do coincide with the natural occurring conditions in the life history of the American eel.

V

DISCUSSION

DISCUSSION

Four in vitro techniques have been employed to study liver metabolism as described in Chapter 3, with the perfused liver and the isolated hepatocyte preparations being preferred for rat liver biochemical studies. Both demonstrate similar quantitative and qualitative results, suggesting that these preparations function in a manner approaching an in vivo situation (Krebs et al., 1973; Shimassek et al., 1973).

The American eel hepatocyte preparation used in this study appears to behave in many respects like the rat hepatocyte preparation. Seglen (1972) reported that the yield of cells, the K^+ content and the oxygen consumption of hepatocytes prepared with a 1 mM Ca^{++} -containing perfusate were higher than hepatocytes prepared with a Ca^{++} -free perfusate. Similar observations were found here (Table III-1 and III-4) and the increase in eel hepatocyte oxygen consumption when prepared with the 1mM Ca^{++} -containing perfusate may be related to a higher mitochondrial Ca^{++} content. Both bovine albumin and gelatine had no effect on the ionic composition, oxygen uptake in the absence of substrate, and membrane intactness of eel hepatocytes (Table III-3 and III-4), but they do increase medium glucose turnover and the glucose stimulation of oxygen consumption (Fig. III-1).

In general, when eel livers were perfused with a 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4, and hepatocytes incubated in the standard Ca^{++} -containing phosphate buffer, pH 7.4, membrane intactness and numbers of cells remained constant over a 2 hr incubation, and the ionic composition (Na^+ , K^+ , Ca^{++}), protein content and the adenylate charge did not differ significantly from intact liver concentrations before and during a 2-3 hr incubation. Major differences, however, were a 35% loss in glycogen

content and higher concentrations of glucose-6-phosphate and pyruvate in eel hepatocytes than in the intact liver (Tables III-1-4).

Consequently, as with the rat hepatocyte, the eel hepatocyte preparation appears to approach an in vivo situation and to be a good technique to study gluconeogenesis in vitro. If the eel and rat hepatocyte systems are compared, however, it is apparent that even though qualitatively the gluconeogenic pathways are similar, major differences in the pattern of control and changes in rates of gluconeogenesis occur, which could represent adaptations to different diet, length of starvation and types of environmental stresses, such as temperature. In this study, eel liver gluconeogenesis was investigated during both a fed (Chapter 3) and starved (Chapter 4) conditions.

The natural diet of a carnivorous fish is high in protein but low in carbohydrate. Although Cowey (1975) showed that fish have a higher protein requirement than mammals, Cowey et al. (1975) demonstrated that fish grow better on a high protein diet containing both fat and carbohydrate than fish fed a high protein diet without carbohydrate. This was due to a higher efficiency and utilization of protein for growth in the presence of carbohydrates. The glucose quantity required for growth may be minimal, but it is necessary.

The exact role of glucose in fish metabolism, however, is still obscure. In general, aerobic energy production is driven by the oxidation of fat, protein and carbohydrate while anaerobic energy production is only from carbohydrate. In fish, fat oxidation was shown to predominate at sustained swimming speeds with glycogen depletion and lactate accumulation following high swimming speeds (Black et al., 1961; Bilinski, 1974). During starvation or migration, however, protein and fat must be

considered as the major fuel source in fish. In the European eel (Dave et al., 1975), fat stores are rapidly depleted within the first month of food deprivation at 5°C while glycogen values are unchanged after 3 months. In many salmonid species, carbohydrate stores are maintained high (Fontaine and Hatey, 1953) or increase (Chang and Idler, 1960) during migration.

Fish carbohydrate may remain constant during starvation or migration if the aerobic energy production from glucose is low, as has been demonstrated by a low glucose oxidation rate in the plaice, Pleuronectes platessa (Cowey et al., 1975) and the carp, Cyprinus carpio (Creach and Murat, 1974). Anaerobic energy production, on the other hand, might be high in fish as shown by glycogen depletion and lactate accumulation during swimming and the relatively high tissue lactate values (Phillips and Hird, 1977). These observations agree with Bennett (1978) who has reported that lower vertebrates rely more on anaerobic metabolism than higher vertebrates.

Consequently, from the above two major functions could be proposed for fish gluconeogenesis; 1) during the fed or starved condition, liver gluconeogenesis recycles glucose from muscle lactate to prevent excessive carbohydrate loss; and 2) during food ingestion, liver gluconeogenesis converts the excess glycerol and amino acids (especially alanine) to glucose (or glycogen) to replace oxidized glucose (or glycogen). This latter function in fish is necessitated by its low carbohydrate diet.

The data reported in this thesis support both functions. At low substrate concentrations, which are probably physiological, lactate is the most effective gluconeogenic substrate during either the fed or food deprived state (Figs. III-7, IV-4 and IV-5). Differences in lactate

gluconeogenic rates during these two nutritional conditions emphasize the importance of glucose recycling. In the fed state, metabolized glucose could be replaced by digested carbohydrates and lactate gluconeogenic rates would be maintained at a low level. During food deprivation, glucose oxidation would decrease and lactate gluconeogenesis increase to maintain total carbohydrate levels (Figs. IV-4 and IV-5). A decrease in glucose oxidation during starvation was reported in the carp (Creach and Murat, 1974).

Glycerol and alanine, at high concentrations, were more effective as gluconeogenic substrates than lactate which is the reverse situation observed at low substrate concentrations (compare Figs. III-3 and III-6). These high concentrations (10 mM) are not physiological (Cowey et al., 1977b), but the trend in effectiveness of gluconeogenic substrates as their concentrations increase, such as during food ingestion, agrees with the second role of gluconeogenesis. It is likely that most of the amino acids, including alanine, are directed towards protein synthesis for growth (Cowey et al., 1975), but a fraction of the excess alanine (serine and proline might also be important since they are good substrates in rat liver (Ross et al., 1967 a, b) is probably utilized by the gluconeogenic pathway to provide the missing dietary glucose (as suggested by Cowey et al., 1977 a) and/or to replace the oxidized glucose. Similarly, the glycerol derived from lipid digestion is probably re-incorporated into lipid in the liver, but the excess could be used for glucose synthesis. Unfortunately, the exact physiological importance of these conversions to glucose remains unknown since neither substrate concentrations in the portal vein during food ingestion nor gluconeogenic rates between low and high substrate concentrations were estimated. Since the rate of glucose

utilization is lower in fish than in mammals (Cowey et al., 1975; Creach and Murat 1974), it might also be expected that the gluconeogenic rate in the eel hepatocyte and rainbow trout liver slice (Cowey et al., 1977a) is lower than rat hepatocyte. The requirement for glucose in the diet of fish may then be explained since in its absence more amino acids are used for gluconeogenesis and less become available for growth.

Amino acid gluconeogenesis during prolonged starvation, however, is different. During starvation, the oxygen consumption decreased in white sucker and trout (Beamish, 1964), and glucose oxidation decreased in carp (Creach and Murat, 1974). Nyman (1972) reported that the activity of European eels was reduced as temperature decreased, suggesting a lower lactate production in skeletal white muscle. Also, energy production from lactate oxidation during starvation in eel hepatocytes was reduced to the minimum required from lactate gluconeogenesis (Fig. IV-9). Thus, the requirement for net glucose synthesis from non-carbohydrate precursors is reduced, explaining the reduced gluconeogenic rates from alanine and aspartate during most of the starvation period at 5°C which represents the length of natural winter fast. The increase in lactate gluconeogenesis appears to be sufficient to maintain total carbohydrate levels in eel.

During the 2nd month at 5°C and 15°C and the 8th month at 15°C, however, amino acid gluconeogenesis increased significantly (Figs. IV-4 and IV-5). Concomitantly, liver glycogen stores were significantly depleted. Therefore, these periods are marked by an enhanced glucose demand. The exact reason for these changes is unknown, but amino acid gluconeogenesis as well as glycogenolysis probably increased because lactate gluconeogenesis was insufficient to satisfy the higher glucose demand.

Consequently, the main task of gluconeogenesis is to provide adequate quantities of glucose (muscle glycogen) required for anaerobic energy production in the eel. The most effective substrate appears to be lactate, the end-product of anaerobic metabolism, suggesting an important role for the Cori cycle in fish. Net synthesis of glucose from glycerol and amino acids is important following food digestion or when there is a very high glucose demand. These conclusions lead to two important questions: How is the gluconeogenic pathway controlled? and, What is the importance of glycogenolysis as a source of glucose?

Although few experiments were done regarding the control of gluconeogenesis in the eel hepatocyte, some tentative proposals can be made. In the fed eel, gluconeogenesis would appear to be controlled by 1) substrate availability since as the substrate concentration increases, rates increase and 2) the glucose amount present in the diet since alanine gluconeogenesis was suppressed in rainbow trout fed a high carbohydrate diet (Cowey et al., 1977a). Since glucose does not appear to stimulate insulin release in fish (Palmer and Ryman, 1972; Cowey et al., 1977a), enhanced dietary glucose levels may prevent glucagon and/or cortisol release which would prevent gluconeogenesis. This effect would be expressed as a decrease in gluconeogenic enzyme activities and ultimately reduced glucose synthesis from all precursors.

During food deprivation, the situation seems to be altered although there is no exogenous glucose source, high blood glucose values were observed and high gluconeogenic rates from lactate were estimated in eel hepatocyte. It was proposed that glucagon is an important hormone during this starvation period even though it increases only lactate gluconeogenesis, not amino acid gluconeogenesis, in fed (Fig. III-7) and late starved

(Table IV-1) eel hepatocytes. Cortisol would be involved only when glucose demand exceeds that available such as the 2nd month. Based on cortisol effects in mammals (Einsenstein, 1969; Kendysh and Moroz, 1972), cortisol would enhance amino acid gluconeogenesis by stimulating gluconeogenic enzyme and transaminase activities and synthesis in eel liver.

The second question concerning the role of glycogenolysis as a glucose source is harder to answer. Starvation or hypoglycemia, in the rat are associated with an initial depletion of glycogen stores, followed by an increase in gluconeogenic rates (Cahill, 1976); i.e., glycogenolysis appears to be the major source of glucose until the glycogen stores are depleted. In contrast to mammals, and although total glucose release from eel hepatocyte glycogenolysis in the absence of substrate (Table III-6) is similar to that reported in rat hepatocyte (Garrison and Haynes, 1973), gluconeogenesis in fish is preferrently increased instead of glycogenolysis during food deprivation or hypoglycemia preventing depletion of carbohydrate stores. The evidence is numerous: 1) during starvation, liver glycogen is maintained (Dave et al., 1975) or only partially depleted, (Stimpson, 1965; Creach and Murat, 1975; Ince and Thorpe, 1976); 2) glucagon stimulates significantly eel hepatocyte gluconeogenesis from lactate (Fig. III-7, Table IV-1), but was unable to stimulate glycogenolysis in fed eel hepatocytes (Fig. III-6) or Japanese eel liver, after injection in vivo (Inui and Yokote, 1977); 3) cortisol enhances gluconeogenesis (Fig. III-5) and glycogenesis, not glycogenolysis in the American eel (Butler, 1968); and 4) good gluconeogenic precursors, such as lactate, glycerol and alanine, prevent glucose release from glycogenolysis in eel hepatocyte (Table III-6). This last evidence really

demonstrates the importance of gluconeogenesis over glycogenolysis.

Since glucose was rapidly converted to glycerol (Fig. III-1 and Table III-5) and that glycerol, per se, reduced glycogen breakdown (Table III-6), it has been proposed that high glycerol concentrations and/or effective glycerogenic substrates (or intermediates), could also inhibit glycogen breakdown. Consequently, glycogen could serve as a source of glucose and glycerol in the American eel.

Only epinephrine has been shown to stimulate glycogenolysis in goldfish hepatocytes (Birnbaum et al., 1976). Since epinephrine is released into the plasma under stress, it could be suggested that glucose production from liver glycogen gains importance only under conditions of stress, such as the 2nd month of starvation in the American eel (Figs. IV-4 and IV-5).

Although the preferred metabolic route for lactate, glycerol, alanine and aspartate was the gluconeogenic pathway (Figs. IV-6,7,8), all these substrates were also found in the glycogen, lipid and CO₂ fractions of the eel hepatocyte. Glycogenesis was the least important pathway for all these substrates probably because, as in the rat hepatocyte (Walli, 1975), good glycerogenic rates in the eel hepatocyte would occur only if non-physiological substrate concentrations are employed and/or there is a low net flux between glucose and glycogen, as proposed by Cowey et al., (1977b). Nevertheless, during some starvation periods, substrate incorporation into glycogen increased in eel hepatocytes (Figs. IV-4,5) suggesting that the relatively constant level of liver glycogen is maintained by a low utilization rate and minor increase in glycerogenic rates.

An important metabolic fate for these substrates was the synthesis of glycerol for triglyceride synthesis. Interestingly, no fatty acids were produced from these substrates (Table III-7) which agrees with the

absence of citrate cleavage enzyme and malic enzyme activities in eel liver (Aster and Moon, 1977). True rates of glycerol production and incorporation into lipid were probably underestimated because of a low fatty acid availability under these experimental conditions. A similar result has been observed in rat adipocytes (Shrago et al., 1978).

In contrast to mammals (Garrison and Haynes, 1973); Shrago et al., 1978), both glycerol esterification and gluconeogenic rates are high in fed eel hepatocytes and increase during starvation which is evidence for the lack of a reciprocal relationship between lipogenesis and gluconeogenesis as reported in mammals (Nikkila, 1973). Moreover, gluconeogenesis is important, not only for glucose production, but also for glycerol production since glucose is not readily available for glycerol production under any nutritional state. Therefore, gluconeogenesis must provide adequate quantities of both glucose and glycerol if glycogen breakdown is to be prevented.

Substrate incorporation in $^{14}\text{CO}_2$ indicated that the substrates, per se, were the main energy fuel (Fig. III-9) and not fatty acids as demonstrated in mammals (Zaleski and Bryla, 1977). Interestingly, oxidation rates were reduced to the minimum required to produce adequate ATP to sustain the observed gluconeogenic rates. This is probably a starvation strategy in which substrate oxidation is reduced to prevent excessive loss of metabolites via CO_2 and H_2O . Since gluconeogenic and oxidative rates from the substrates used increase during starvation, pyruvate flux through pyruvate carboxylase and pyruvate dehydrogenase must also increase. This is evidence for the lack of a reciprocal relationship in eel hepatocyte mitochondria between these two enzymes as reported in mammals (Walter et al., 1973).

Alanine oxidation shows particularity in fed eel hepatocytes. At low concentration, the rate of alanine oxidation was low and in a similar range to the other substrates. At 10 mM alanine, however, the rate was 7-times greater than the other substrates. Thus, as alanine concentration increases in the portal vein, e.g., during food ingestion, its importance as an energy source also increases relative to other substrates, which is probably an adaptation to the high protein diet of the carnivorous fish. Consequently, the excess alanine (and probably proline, serine and glycine) which is not readily incorporated into body protein, may serve as a substrate for the production of ATP, glycerol and glucose in eel liver during adequate nutritional conditions.

CONCLUSIONS

The data collected in this thesis suggests the following conclusions:

- 1) In contrast to mammals, eel liver gluconeogenesis is preferrently stimulated relative to glycogenolysis to provide adequate plasma glucose.
- 2) The primary function of eel liver gluconeogenesis is the recycling of glucose from lactate, produced by anaerobic metabolism, to maintain constant carbohydrate levels.
- 3) A second function of eel liver gluconeogenesis is a net synthesis of glucose from non-carbohydrate precursors obtained from the diet (fed state) or from skeletal muscle (starved state) to replace metabolized glucose.
- 4) A third function is to provide an adequate quantity of glycerol for lipogenesis. In the absence of effective glycerogenic substrates, glycerol may be produced from glycogen breakdown, but this probably is not a predominant source.
- 5) During starvation, glucose oxidation probably decreases, and the need for net glucose synthesis is reduced. Consequently, amino acid gluconeogenesis is also reduced. The increase in lactate gluconeogenesis appears sufficient to prevent massive liver glycogen breakdown and to maintain blood glucose.
- 6) Both amino acid gluconeogenesis and glycogenolysis are stimulated when lactate gluconeogenesis is insufficient to satisfy a high glucose demand such as during the 2nd month of starvation.
- 7) All gluconeogenic substrates, including alanine, glycerol, aspartate

and alanine, are used to provide the ATP required to sustain their own incorporation into glucose during food deprivation. During food ingestion, however, alanine appears as an important energy source in eel liver.

VI

APPENDIX

1. Variability of the standard enzymic determinations and radioactive determinations (see Methods and Materials) using the eel hepatocyte preparation. Two St. Lawrence eel livers (from eels fed 5 months every day on a beef liver diet, starved 2 months and refed 2 weeks) were perfused with a 1 mM Ca^{++} -containing bicarbonate buffer, pH 7.4, and hepatocytes were incubated 1 hr at $15 \pm 1^\circ \text{C}$ as described in the text, except that the hepatocyte suspension was divided into 6 incubation flasks to which were added 1 mM cold lactate and 1 μCi ^{14}C -lactate.

Variability (in %) is the SEM divided by the mean times 100.

PARAMETERS	Fish A		Fish B	
	Rates (mean $\pm$ sem)	Variability %	Rates (mean $\pm$ sem)	Variability %
<u>Enzymatic measurement</u>				
- Glucose release (moles/100 mg)	6.67 $\pm$ 0.15	2.2%	3.71 $\pm$ 0.08	2.2%
- Hep. Glycogen (mg/100 mg)	5.10 $\pm$ 0.23	4.5%	7.76 $\pm$ 0.25	3.2%
<u>Radioactive counts</u> (nmoles/100 mg)				
- CO ₂	95.1 $\pm$ 3.5	3.6%	128.2 $\pm$ 11.6	9.0%
- Lipid	70.8 $\pm$ 2.0	2.8%	80.2 $\pm$ 2.1	2.6%
- Glycogen	10.6 $\pm$ 0.8	8.1%	4.5 $\pm$ 0.5	10.7%
- Glucose	548.3 $\pm$ 29.7	5.4%	301.2 $\pm$ 24.9	8.2%
<u>Ratio</u>				
Glycogenic : Lipogenic	0.15 $\pm$ 0.01	9.2%	0.06 $\pm$.01	13.1%
Gluconeogenic : Glycogenic	53.2 $\pm$ 5.2	9.5%	67.6 $\pm$ 4.2	6.1%
Gluconeogenic : Lipogenic	7.7 $\pm$ 0.3	4.0%	3.7 $\pm$ 0.4	9.3%

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