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CARBOHYDRATE METABOLISM AND THE ROLE OF INSULIN AND GLUCAGON IN ISOLATED HEPATOCYTES OF THE SEA RAVEN *Hemitripterus americanus* Gmelin

by

Glen D. Foster

A Thesis presented to the University of Ottawa in partial fulfillment of the requirements for the degree of Masters of Science in the Department of Biology

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ABSTRACT

Hepatocytes were isolated from the sea raven, *Hemitripterus americanus* for the *in vitro* metabolic study of insulin and glucagon. The hepatocytes were preincubated 3 h in the presence of the appropriate hormones, rinsed, and the experiments were performed on a final 2 h incubation. A steady, but slow depletion of cell glycogen occurred during incubation. Bovine glucagon at 10^{-8} M increased the depletion rate, while a mammalian (porcine) insulin at 10^{-9} M maintained the glycogen content for 1.5 h. Teleost (swordfish) insulin at 10^{-9} M or 10^{-8} M was without effect. Both teleost insulin and bovine glucagon increased glucose levels, but mammalian insulin did not alter levels from control. Ratios of glucose production to glycogen loss increased from 20% to 29% ($p<0.05$) in the presence of glucagon, decreased from 20% to 9% with mammalian insulin, and increased from 20% to 69% ($p<0.01$) with teleost insulin.

^{14}C-glucose and ^{14}C-serine flux to various endproducts was measured. The sole effect of any of the hormones on ^{14}C-glucose flux was an increase in isotope incorporation into glycogen by glucagon addition (10^{-8} M). Glucagon also increased serine flux to glucose (3 times) and glycogen (2 times), without changing flux to CO_2. The presence of both glucagon (10^{-7} M) and cortisol (10^{-7} M) did not alter serine flux except for a small increase to CO_2. Teleost insulin

(10^{-8} M) increased serine flux to glycogen and lipids, as well as glucose. Mammalian insulin (10^{-9} M) also increased flux to glucose and glycogen, but not to lipids; a significant increase in radioactive protein occurred at 10^{-8} M insulin. An additive effect on serine gluconeogenesis and total glucose production was observed when teleost insulin and mammalian glucagon were both present in the incubations. However, an antagonism was apparent with respect to serine flux to glycogen and CO_2. Glucagon increased the V_{opt} of sea raven hepatocyte phosphoenolpyruvate carboxykinase, and decreased the V_{opt} of both phosphofructokinase and pyruvate kinase, demonstrating a direct effect of this hormone on the gluconeogenic pathway. Teleost insulin was without effect on the three enzymes tested, while mammalian insulin only decreased the activity ratio of PFK, inferring an increase in the enzyme-substrate affinity. Fructose 2,6-bisphosphate increased both the V_{opt} and activity ratio of PFK.

These results demonstrate that in sea raven hepatocytes incubated *in vitro*, glucagon functions to increase glucose production through gluconeogenesis and glycogenolysis. Insulin increases carbohydrate production from an amino acid precursor. It is suggested that glucagon functions to increase glucose production from energy stores and gluconeogenic precursors obtained from muscle

metabolism during periods of food-deprivation. Insulin may increase liver glucose production for export to, and storage in, glucose-utilizing tissues, and also increase liver energy stores, from amino acids obtained from feeding.

RESUME

Des hépatocytes ont été isolées de l'hémitriptère atlantique, *Hemitripterus americanus* afin d'etudier le métabolisme quant à l'insuline et le glucagon *in vitro*. Après une période de préincubation de 3 h en présence des hormones appropriées, suivie d'un rinçage, les hépatocytes ont été soumises à des manipulations au cours d'une dernière période d'incubation d'une durée de 2 h. Un abaissement lent et continu de glycogène cellulaire se produit pendant l'incubation. Ce taux d'abaissement augmente en présence du glucagon bovin (10^{-8}) tandis que l'insuline pocine (10^{-9}) maintient les niveaux de glycogène pour une période de 1.5 h. Aucune des deux concentrations de l'insuline de l'espadon *Xiphias gladius* soit 10^{-9} M et 10^{-8} M, n'affecte les niveaux de glycogène. Les niveaux de glucose subissent une hausse en présence du glucagon bovin et de l'insuline de *Xiphias* mais n'enregistrent aucun changement suite à l'addition d'insuline porcine. Le rapport entre la production de glucose et le perte de glycogène augmente de 20% à 29% ($p<0.05$) en présence du glucagon, diminu de 20% à 9% en présence de l'insuline porcine, et augmente de 20% à 69% ($p<0.01$) en présence de l'insuline de *Xiphias*.

Les flux de ^{14}C-glucose et de la ^{14}C-sérine vers qulques produits hépatiques ont été déterminés. Le flux de ^{14}C-glucose répond positivement

à l'addition du glucagon mais n'enregistre aucun changement en présence des autres hormones. Le glucagon (10^{-8} M) augmente donc l'incorporation de ces isotopes au glycogène. Le glucagon augmente aussi le flux de la serine au glucose (3 fois) et au glycogène (2 fois) sans affecter le flux au CO_2. L'addition du glucagon (10^{-7} M) et du cortisol (10^{-7} M) a pour seul effet une minime augmentation en CO_2. L'insuline de *Xiphias* (10^{-8} M), augmente l'incorporation de la sérine dans le glycogéne, les lipides et aussi le glucose. L'insuline porcine (10^{-9} M), augmente aussi le flux de la sérine au glucose et au glycogéne, mais non aux lipides; à une concentration de 10^{-8} M l'insuline produit une hausse significative de la radioactivité des protéines. La présence simultanée de l'insuline de *Xiphias* et du glucagon mammifère prduit un effet additif sur la gluconéogénèse de la sérine et sur la production totale de glucose. Toutefois, le flux de la sérine au glycogène et au CO_2 dénote de façon évidente l'occurence d'un antagonisme. Le glucagon augmente la V_{opt} de la phosphoénolpyruvate-carboxykinase dans les hépatocytes de l'hémitriptère, et diminu la V_{opt} de la phosphofructokinase (PFK) et de la pyruvate-kinase. Le glucagon affecte donc la gluconéogénèse directement. L'insuline de *Xiphias* n'affecte pas l'activité de ces trois enzymes. Par contre, l'insuline porcine diminue le

rapport de l'activité de la PFK, ce qui démontre une plus grande affinité entre l'enzyme et sons substrat. La fructose 2,6-bisphosphate élève la V_{opt} ainsi que le rapport de l'activité de la PFK.

Ces résultats démontrent que chez L'hémitriptère, le glucagon augmente la production de glucose au moyen de la gluconéogènése et de la glycogénolyse dans les hépatocytes incubées *in vitro*. L'insuline augmente la production des hydrates de carbone à partir d'un substrat gluconéogènique.

Il est proposé qu'en période de jeune, le glucagon obtient du métabolisme musculaire les substrats gluconéogèniques et les réserves d'energie nécessaires à provoquer une hausse de la production de glucose. Il est aussi proposé que l'insuline augmente la production hépatique de glucose pret à l'exportation et à l'emmagasinage dans les tissus qui utilisent le glucose et augmente aussi les réserves hépatiques d'energie à partir d'acides aminées obtenues dans la nourriture.

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LIST OF ABBREVIATIONS

cAMP	cyclic adenosine 5'monophosphate
DTT	dithiothreotol .
E.ase	enolase (EC 4.2.1.11)
F2,6-P	D-fructose 2,6-bisphophate
F6-P	D-fructose 6-phosphate
FBPase	D-fructose 1,6-bisphoshatase (EC 3.1.3.11)
FBPase 2	D-fructose 2,6-bisphosphatase
FRM	final resuspension medium
GDH	D-glycerate dehydrogenase (EC 1.1.1.29)
GK	D-glycerate kinase (EC 2.7.1.31)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
K_m	Michaelis-Menton constant
LDH	L-lactate dehydrogenase (EC 1.1.1.27)
MDH	L-malate dehydrogenase (EC 1.1.1.37)
NADH	nicotinamide adenine dinucleotide,reduced form
PC	pyruvate carboxylase (EC 6.4.1.1)
PCA	perchloric acid
PDH	pyruvate dehydrogenase
PEPCK	phosphoenolpyruvate carboxykinase (EC 4.1.1.31)
PFK	phosphofructokinase (EC 2.7.1.11)
PK	pyruvate kinase (EC 2.7.1.40)
SDH	serine dehydratase (EC 4.2.1.13)
V_{max}	maximal activity
V_{opt}	optimal activity

CHAPTER 1:

INTRODUCTION

Insulin and glucagon are polypeptide hormones produced by the islet cells of vertebrates which act on target tissues through specific membrane interactions (Turner and Bagnara, 1976). The two hormones are structurally dissimilar. Insulin has a molecular weight of 6000, and is composed of 51 amino acid residues in two polypetide chains connected by two disulphide bridges. Glucagon has a molecular weight of 2800, and is a single polypeptide chain of 29 amino acid residues. Insulin is evolutionarily ancient, with insulin-like molecules and islet-like cells being found in prechordate organisms; insulin itself, and B cells from which it is produced, are present in all agnathans and gnathostomes examined to date (Epple, 1969). Glucagon has not been found in organisms more primitive than elasmobranchs. Insulin and glucagon from various vertebrate species show differences with respect to biological activity and amino acid sequences (Epple, 1969).

The islets of Langerhans, or Brockman bodies, of teleosts are structurally and chemically similar to those found in mammals. Insulin is synthesized in the B cells and glucagon in the A cells, high levels of zinc are present in the tissue, and the exocrine pancreas is associated with the islets (Epple, 1969). The islet cells are freely

permeable to glucose in those fish examined, and the major carbohydrate-metabolizing pathways are present (the Embden-Meyerhof pathway and the pentose phosphate shunt) (Epple, 1969). Such data have been used to support a role for glucose in insulin and glucagon secretion. A major anatomical difference from mammals is that the pancreas of most teleosts is loosely scattered throughout the body cavity and organs.

The effects of insulin and glucagon have been extensively studied in mammals and are well-defined, both on whole animal and on liver metabolism. Biochemically the two hormones have antagonistic functions. In general, insulin in whole animal metabolism is anabolic (Reiser, 1967), while glucagon is catabolic (Unger and Orci, 1981) (Table 1-1). Insulin secretion is stimulated primarily by glucose (Turner and Bagnara, 1976) and it acts to decrease blood glucose through enhancing glucose uptake into muscle and adipose tissues, and by increasing glucose oxidation and glucose flux to lipid, protein, and carbohydrate stores. The liver is freely permeable to glucose (Guyton, 1977). Glucagon release is stimulated, in part, by low glucose levels, and it increases blood glucose levels through the mobilization of tissue energy stores and their subsequent conversion to glucose (Unger and Orci, 1981).

The mammalian liver functions as a "glucostat" by finely controlling glucose utilization or production through the effects of hormones which include insulin and

glucagon. The points of action of insulin and glucagon are diagrammed in Fig. 1-1 and 1-2, respectively. The two hormones affect the metabolic machinery by changing amino acid uptake and by altering enzyme activities, either by post-translational (phosphorylation) or genomic mechanisms. Dephosphorylation of glycogen synthetase by insulin results in its conversion from the inactive "b form" to the active "a form" and glycogen phosphorylase from the "a" to the "b" form through a protein kinase (Witters and Avruch, 1978). Glycolysis is stimulated by the allosteric activation of phosphofructokinase (PFK) (Pilkis et al., 1983) and deactivation of fructose 1,6-bisphosphatase (FBPase) (Pilkis et al., 1981). These changes are effected by the deactivation, through dephosphorylation, of phosphofructokinase 2 (PFK 2) and fructose 2,6-bisphosphatase (FBPase 2), and the resulting decrease in F2,6-P levels which antagonistically alters PFK and FBPase activities. Pyruvate kinase (PK) is dephosphorylated by an insulin stimulation of a protein phosphatase, resulting in decreased activity (Feliu et al.,1976; Parks and Drake, 1982), while the same mechanism is possibly used to increase the activities of pyruvate dehydrogenase (PDH) (Wienberg and Utter, 1980) and acetyl CoA carboxylase (Witters, 1981). Genomic effects of insulin are reported, where the synthesis of glucokinase (Weber, 1971), PK (Parks and Drake, 1982), and fatty acid synthetase (Pry and

Table 1-1: Insulin and glucagon effects on various pathways of the rat liver (Reiser, 1967; Unger and Orci, 1981).

PATHWAY	INSULIN	GLUCAGON
GLYCOLYSIS	+	–
GLUCONEOGENESIS	–	+
LIPOLYSIS	–	+
LIPOGENESIS	+	–
PROTEIN SYNTHESIS	+	+

Fig. 1-1: Some points of action of insulin on gluconeogenesis and glycolysis in the rat liver. 1) membrane transport, 2) glucokinase, 3) glucose-6-phosphatase, 4) glycogen synthetase, 5) glycogen phosphorylase, 6) phosphofructokinase, 7) fructose bisphosphatase, 8) pyruvate kinase, 9) phosphoenolpyruvate carboxykinase, 10) pyruvate carboxylase. +, increased activity; -, decreased activity.

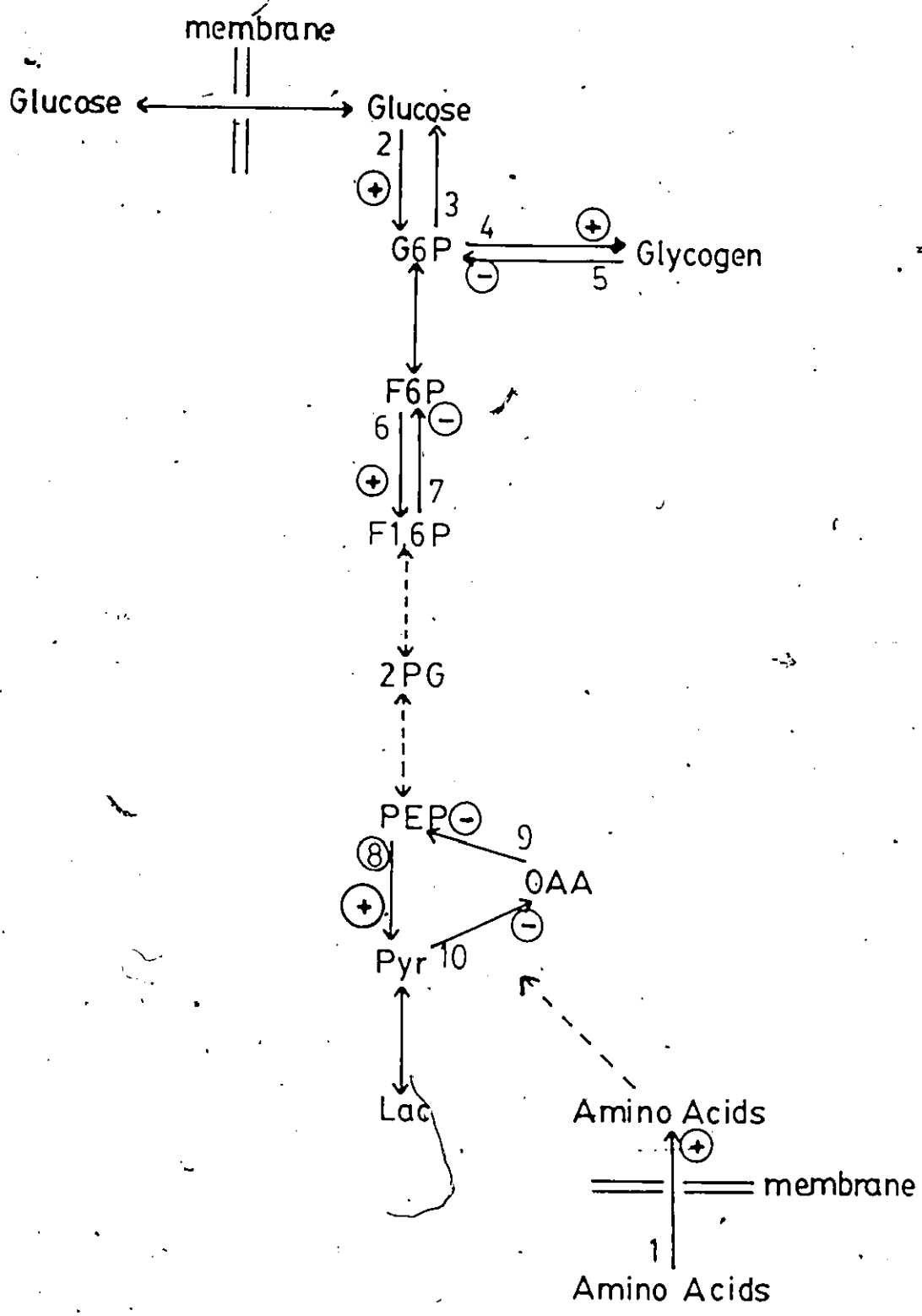
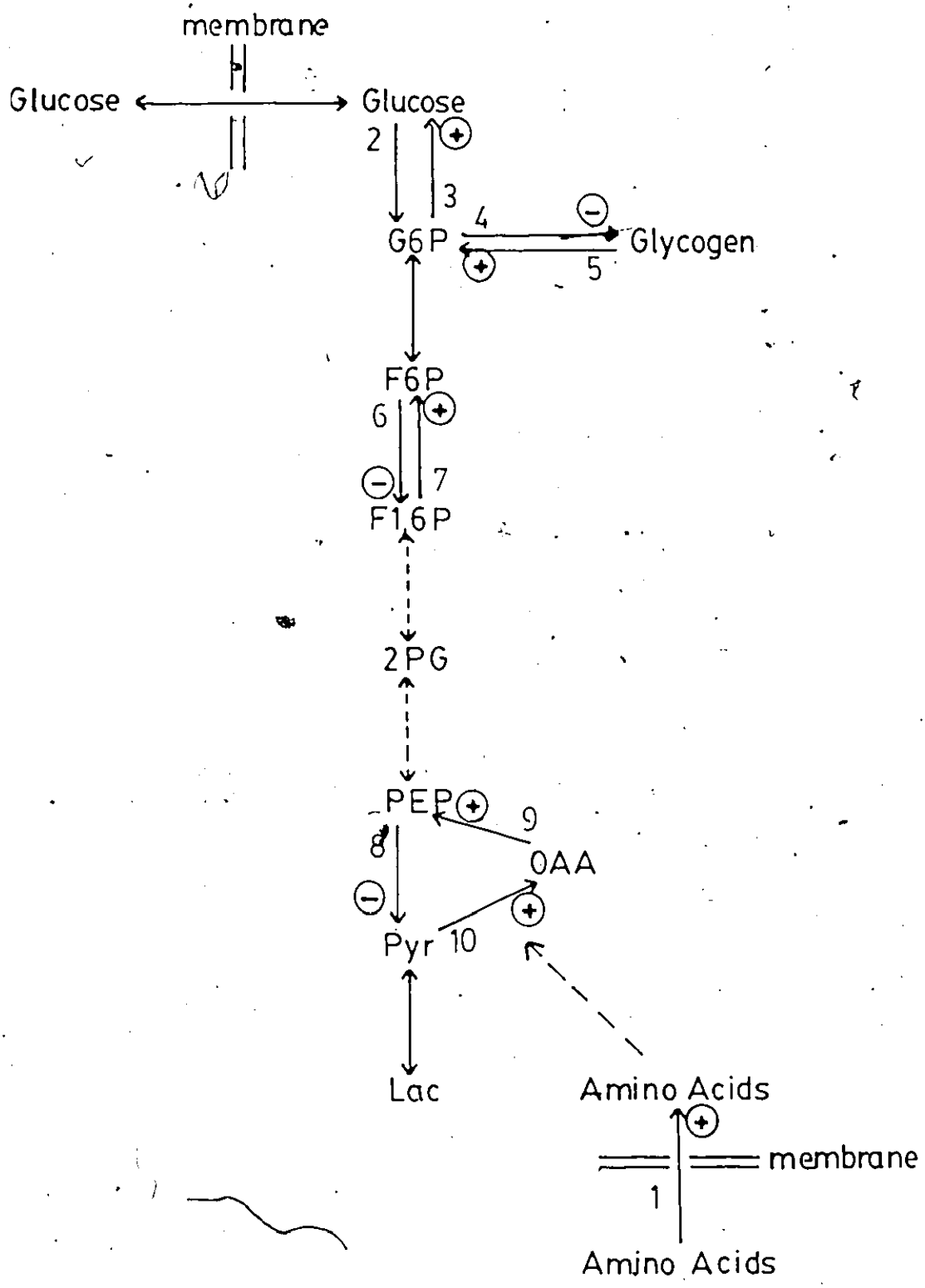

membrane
Glucose
Glucose
2
3
G6P
4
5
Glycogen
F6P
6
7
F1,6P
2PG
PEP
9
8
OAA
Pyr
10
Lac
Amino Acids
membrane
1
Amino Acids

Fig. 1-2: Some points of action of glucagon on gluconeogenesis and glycolysis in the rat liver. Legend as in Fig. 1-1.


membrane
Glucose
Glucose
2
3
4
5
G6P
Glycogen
F6P
6
7
F1,6P
2PG
PEP
9
8
OAA
Pyr
10
Lac
Amino Acids
membrane
1
Amino Acids

Porter, 1981) are stimulated, while pyruvate carboxylase (PC) (Weinberg and Utter, 1980), and phosphoenolpyruvate carboxykinase (PEPCK) synthesis (Cimbala et al., 1981; Felig, 1981; Sasaki et al., 1984) is decreased.

Glucagon effects generally antagonize those of insulin. It increases phosphorylation mechanisms, resulting in opposite effects on glycogen synthetase and phosphorylase (Exton, 1981), PFK and FBPase (Pilkis et al., 1981, 1983), and PK (Feliu et al., 1976; Pilkis et al., 1981). It acts genomically to increase the activity of PEPCK (Cimbala et al., 1981; Sasaki et al., 1984; Iynedjian et al., 1985) and PC (Felig, 1981), and by decreasing the activity of fatty acid synthetase (Pry and Porter, 1981). PDH activities have been reported to decrease after glucagon exposure (Oviasu and Whitton, 1984). By altering enzyme activities, insulin and glucagon direct the movements of specific metabolites in the liver and thus decrease or increase glucose production in a manner consistent with the glucostatic function of the mammalian liver. Insulin effects on the gluconeogenic pathway act only to decrease the glucagon-stimulated increases (Hue, 1982), whereas insulin can act on the basal levels of other pathways. Both insulin and glucagon increase amino acid uptake into the rat hepatocyte (Freychet et al., 1979). Insulin increases protein synthesis (King and Kahn, 1981) while glucagon increases gluconeogenesis from amino acids

(Freychet et al., 1979). Protein degradation is inhibited by insulin and stimulated by glucagon (Poli et al., 1981). Some protein effects such as growth may occur through a receptor which is different from that responsible for the metabolic effects of insulin (King and Kahn, 1981).

The role of insulin anf glucagon in the liver of teleosts is less defined than in mammals. Insulin administration alters glucose and amino acid contents in the blood of various teleost fishes as in mammals (e.g. Minick and Chavin, 1970; Larsson and Lewander, 1972; Ince and Thorpe, 1975, 1976, 1978; Inui and Yokote, 1975; Murat and Serfaty, 1975; Lewander et al., 1976; Murat et al., 1978; Ablett et al., 1981a,b; Carniero and Amaral, 1983), but the changes in liver metabolism are ambiguous. For example, insulin increases, decreases, or does not change liver glycogen content in a variety of teleosts (Gray, 1928; Leibson and Plisetskaya, 1968; Inui and Yokote, 1975; Murat et al., 1975; Lewander et al., 1976; Carniero and Amaral, 1983). Similarily, diverse effects are noted on the hepato-somatic index (Inui and Yokote, 1975; Lewander et al., 1976; Ablett et al., 1981a; Carniero and Amaral, 1983) and protein and lipid levels (Inui and Yokote, 1975; Lewander et al., 1976; Ablett et al., 1981a; Carniero and Amaral, 1983; Sower et al., 1985). The flux of ^{14}C-glucose and ^{14}C-amino acids to various endproducts in the pike and trout are also inconsistent (Ince and Thorpe, 1976; Ablett et al., 1981a,b). Glucagon

has received less study than insulin, but an increase in blood glucose levels is generally reported (Tashima and Cahill, 1964; Plisetskaya, 1972; Larsson and Lewander, 1972; Chan and Woo, 1978; Castilla et al., 1979; Carniero and Amaral, 1983) coupled with inconsistent changes in liver glycogen (Murat and Plisetskaya, 1977; Chan and Woo, 1978; Carniero and Amaral, 1983). The reason for the great diversity of responses to these peptide hormones is unknown, although species and dose differences are obvious factors. Unfortunately, no specific method is yet available to quantify either hormone in the blood of a teleost, a factor slowing research in this field (e.g. Sower et al., 1985).

Whole animal studies suffer from the inability to control a variety of variables, such as hormone feedback mechanisms and the complex interactions between hormones. More recently, isolated tissue studies have become the preferred tools for hepatic studies (Hayashi et al., 1982; Campbell et al., 1983; Moon et al., 1985). Such studies indicate a general increase in glycogen levels in insulin-exposed hepatocytes of the salmon (Plisetskaya et al., 1984) and the perfused catfish liver (Ottolenghi et al., 1985), with increased glucose incorporation occuring in the latter species. Renaud and Moon (1980) reported decreased medium glucose levels, with no change in radioactive glucose flux to CO_2, lipid, or glycogen in eel hepatocytes. These findings demonstrate an overall

anabolic effect of insulin, but the resultant changes in metabolite fluxes are as yet poorly defined. For instance, no fish hepatocyte studies to date have examined insulin effects on gluconeogenesis and amino acid and lactate compartmentalization. The mechanistic changes occurring with insulin in the liver also remain obscure.

Glucagon-exposed hepatocytes generally increase total glucose production and gluconeogenesis from lactate and amino acids (Tashima and Cahill, 1964; Walton and Cowey, 1979a; Renaud and Moon, 1980; Morata et al., 1982; Mommsen and Suarez, 1984) while glycogen levels decreased in toadfish liver slices (Tashima and Cahill, 1964). Whether glycogen plays a part in the increased glucose production *in vitro*, however, has not been determined directly. Effects on enzymes are unknown, except for one study which found an increase in the apparent K_m of pyruvate kinase in trout hepatocytes exposed to glucagon (Mommsen and Suarez, 1984). While insulin effects in the teleost liver remain unclear, it is apparent that glucagon increases total glucose production through increased gluconeogenesis and, possibly, glycogenolysis.

Teleost liver carbohydrate metabolism is distinct from that of mammals. For instance, the teleost liver has no glucokinase, only a low K_m hexokinase (Ureta, 1981), and the tissue exhibits low glucose utilization. The tissue, however, has a significant gluconeogenic function (Cowey and Sargent, 1979; Moon et al., 1985). This apparent

unidirectionality of teleost liver metabolism may prevent it from functioning as a glucostat and preclude any profound antagonism between insulin and glucagon as is seen in mammals.

Insulins from diverse species are found to have differential effects on liver metabolism. Codfish insulin decreased blood glucose, cholesterol, and amino acids in the European eel, whereas bovine insulin decreased only the amino acid content (Ince and Thorpe, 1974). In the pike, codfish insulin decreased free fatty acid levels, while bovine insulin was without effect (Ince and Thorpe, 1975). Only high levels of bovine insulin caused a lowering of blood glucose in this fish (Thorpe and Ince, 1974). In the goldfish, however, a variety of insulins, including bovine and teleost, decreased blood glucose levels at relatively low doses (Minick and Chavin, 1970). The differential effects of insulins are thought to be a function of the primary structure of these proteins (Epple, 1969). Little is known about the differential effects of glucagon from different species or of the amino acid sequences of these molecules.

Lastly, no studies to date have examined insulin and glucagon effects on the isolated hepatocytes of a single teleost species and, therefore, the degree of antagonism which may exist between the two hormones in fish is not known.

The aim of this thesis is to establish the

consequences of insulin and glucagon administration on the metabolism of a teleost hepatocyte system. Specifically, the project addresses a number of questions related to the function of the individual hormones and their inter-related activities:

1. are the effects of glucagon in sea raven hepatocytes similar to the effects in other teleosts?
2. is glycogen, as well as gluconeogenic precursors, a substrate for the observed glucagon-enhanced glucose production?
3. how does insulin alter metabolite movements and enzymes in a teleost liver?
4. does antagonism exist between glucagon and insulin in a teleost liver?
5. are there differential effects of mammalian and teleost insulins in teleost hepatocytes?

The methodology involves the *in vitro* estimates of glucose production and utilization, changes in glycogen content, glucose and amino acid fluxes, and selected enzyme characteristics of hepatocytes isolated from the sea raven, *Hemitripterus americanus*. This species is suited for such experiments because of its large liver size, the ease of obtaining large numbers of cells, the relative stability of liver glycogen levels, and the availabilty of some background knowledge of this species (Milligan and Farrell, 1985; Walsh et al., 1985). The sea raven belongs to the

family Cottidae and is a "sit-and-wait" predator that feeds easily under laboratory conditions.

A teleost insulin is used in this study to determine the differential effects of various insulins. Few teleost insulins are available, but swordfish insulin was kindly provided by Connaught Laboratories. The insulins from marine teleosts that have been sequenced to date have shown relative residue conservation (Blundell, 1972), thus it is assumed that the actions of the swordfish insulin will reflect the actions of the endogenous insulin of the sea raven. Porcine insulin was also used for comparative purposes. Teleost glucagon is not available, so only bovine glucagon was used in the experiments. The limitations of this protocol must be considered in the interpretation of the results.

CHAPTER 2:

MATERIALS AND METHODS

ANIMALS

Sea ravens, *Hemitripterus americanus*, weighing between 750 and 2000 g were obtained from Passamaquoddy Bay, N.B. by otter trawl and maintained in flowing, unflitered sea water at the Huntsman Marine Laboratory or the Biological Station, Dept. of Fisheries and Oceans, St. Andrews, N.B. Water temperatures were maintained at 10°/+/2° and the salinity varied between 26 and 30°/°°. Animals were held in the laboratory for a minimum of one week. Those held longer than one week were fed live fish weekly, and all animals had food in their gut at the time of sacrifice.

CHEMICALS

All biochemicals were obtained from either Sigma Chemical Co. (St. Louis, MO) or Boehringer-Mannheim Canada Ltd (Lacine, PQ). Radioactive substrates were acidified, and after the solvent was evaporated under a stream of nitrogen, they were dissolved in the final resuspension medium (see below) and frozen at -20°C. Bovine glucagon and porcine insulin were generously provided by Dr. M. Root of Eli Lilly (Indianapolis, IN) and the swordfish insulin was a gift from Dr. J.P. Clement of

Connaught Laboratories (Toronto, Ont.). Starch gel electrophoresis of the swordfish insulin showed a single, diffuse protein band. The hormones were dissolved in 0.001 N HCl to a stock concentration of 1 $mg.ml^{-1}$ prior to dilution in the appropriate saline solution. Hormone solutions were prepared and stored in plastic test tubes. All other reagents were obtained from local suppliers and were of the highest possible purity.

HEPATOCYTE PREPARATION

All hepatocyte preparations were begun between 8:00 and 9:30 am to avoid the posibility of diurnal changes in hormone levels (Gutierrez et al., 1984). Sea raven hepatocytes were prepared with modifications to the classic technique of Seglen (1976), and modified slightly from Walsh et al. (1985). Blood vessels leading to the liver were cannulated and perfused with a saline solution (Solution A) containing 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 0.6 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 10 mM $NaHCO_3$, and 10 mM HEPES, at pH 7.6 (adjusted with NaOH). After the liver was cleared of blood, the perfusate was changed to solution A containing collagenase (from *Clostridium histolyticum*, Sigma Type 4, 0.4 $mg.ml^{-1}$). Neither heparin nor Ca^{++} were used at any point in the procedure. Perfusion proceeded until the liver had swollen (between 10 and 15 min), whereupon the free cells were gently milked into a beaker.

The cells were filtered through a 180 um nylon mesh and centrifuged at 3000 g for 90 sec. at 5°C. The resultant pellet (hepatocytes) was rinsed 2 times to remove broken cells and red blood cells in Solution A at 3000 g for 90 sec., then resuspended in the final resuspension medium (FRM) containing 140 mM NaCl, 5 mM KCl, 0,8 mM $MgSO_4$, 0.6 mM NaH_2PO_4, 10 mM $NaHCO_3$, 1 mM $CaCl_2$, 10 mM HEPES, and 2% BSA at pH 7.6 (adjusted with NaOH). The final resuspension contained 30 to 60 x 10^6 cells.ml^{-1} or about 40 to 100 mg dry cell wt.ml^{-1}. Walsh et al. (1985) demonstrated a high degree of cell integrity using trypan blue and succinate oxidation measurements. Using the slightly modified method in this study (no heparin, Ca^{++}, or glucose at pH 7.6) the cells consistently exhibited greater than 95% viability by trypan blue exclusion.

EXPERIMENTAL PROTOCOL

Substrate Utilization: The ability of the sea raven hepatocytes to utilize various substrates was estimated by measuring the rates of glucose and CO_2 production from selected [U-^{14}C]-substrates. Hepatocytes (1 ml) were pipetted into 30 ml serum vials containing 1 ml of 5 mM substrate (either lactate, alanine, serine, glycine, aspartate, leucine, or histidine, with their respective [U-^{14}C] radioisotopes at 3.5 to 5.0 x 10^5 DPM per incubation. Incubations proceeded for 2 h while shaking at

10°C and were stopped by injecting 0.3 ml of 0.2 N H_2SO_4 through the rubber stopper. CO_2 and glucose were extracted as described in the Metabolite Analyses section.

Hormone Studies: An initial study determined the effects of hormone concentration on flux. Hepatocytes were incubated in 30 ml serum vials containing either 0, 10^{-9} M, 10^{-8}, or 10^{-5} M bovine glucagon or porcine insulin, or 10^{-9} M or 10^{-8} M swordfish insulin. Both 5 mM glucose and 5 mM serine were present together for a 3 h pre-incubation period at 10°C. Following this period, the cells were rinsed and resuspended in fresh FRM. The final incubations contained the same hormone conditions as initially used, but with either 5 mM glucose or 5 mM serine. For the radioactive studies, 3.5 to 5.0 x 10^5 DPM [U-^{14}}-glucose or -serine were added to the appropriate vials. The final incubations proceeded for 2 h (radioactive studies) or the stated times (metabolite determinations), and were stopped by the addition of 0.2 ml of 30% PCA (glycogen/protein studies) or 0.3 ml of 2 N H_2SO_4 (all other experiments).

In a second experiment, the interactive effects of the hormones were studied. The same protocol was followed, except that glucose was not included in any of the incubations, and the vials contained either (1) 10^{-7} M bovine glucagon, 10^{-7} M cortisol, or both 10^{-7} M

bovine glucagon and cortisol, or (2) 10^{-8} M swordfish insulin, 10^{-7} M glucagon, or both 10^{-8} M insulin and 10^{-7} M glucagon.

METABOLITE ANALYSES

Radioactive Experiments: $^{14}CO_2$ was captured into hyamine hydroxide after acidification of the incubate (NEN Canada Ltd.). The trapping agent (0.15 ml) was injected through the stopper onto a filter paper (Whatman GF/A) in a center well suspended over the incubate. The filter paper was removed after 2 h of shaking and counted in Lipofluor (Baker Chemical Co.).

Following $^{14}CO_2$ extraction, the incubate was quantitatively transferred to a tube and centrifuged at 3000 g for 5 min. The supernatant was frozen at -20°C for future analysis. To determine incorporation into protein, the pellet was rinsed 2 times with 5 ml of 3% PCA, solubilized in 1 ml of NCS (Amersham Canada Ltd.) and 0.5 ml of water. Counting was carried out in 10 ml of Aqualyte (Fisher Scientific).

Glucose was separated by ion exchange methods. The acidified incubation supernatant was passed through a column of Dowex AG-1 x 8 (Cl^- form) to remove negatively charged species (Reilly, 1975). The column effluent was added to a test tube containing equal amounts of Dowex 50 x 8 (H^+ form) and Amberlite IR-4B (OH^- form) to remove positively charged species (Exton

and Park, 1967). An aliquot of the supernatant containing glucose was counted in Aqualyte (Fisher Scientific). Counts were corrected for extraction efficiency (92%) and serine contamination (3%), which were independent of sample pH.

Lactate extraction was accomplished by passing a neutralized (with $KHCO_3$) aliquot of the incubation supernatant through a column of Dowex AG-1 x 8 (Cl^-) form), and the trapped lactate was liberated by adding 0.5 M formic acid to the column. An aliquot of the resulting effluent was added to Aqualyte for counting. Counts were corrected for glucose contamination (7.5%) and collection efficiency (67%).

Glycogen was extracted by ethanol precipitation. Ethanol was added to a final concentration of 60% to the supernatant of the PCA-precipitated incubates, and the glycogen was allowed to precipitate for 12 h at 5°C. The pellet, containing the glycogen, was rinsed 2 times with 95% ethanol, dried under a stream of nitrogen, and redissolved in 1 ml of distilled water. Aliquots were counted for radioactivity (Aqualyte; Fisher Scientific).

Total lipids were extracted according to Bligh and Dyer (1959). An aliquot of the incubation supernatant was homogenized in 2:1 chloroform-methanol (v:v) and filtered through Whatman GF/A flter paper. The filtrate was washed with 0.04% $CaCl_2$ followed by rinsing with 6:96:94 chloroform-methanol-0.04%$CaCl_2$ (repeated 3 times). The chloroform layer was evaporated to dryness under nitrogen

and 10 ml of Lipofluor were added for counting.

All radioactive counting was performed with an LKB Rackbeta 1211 scintillation counter equipped with internal standardization.

Glycogen/Glucose Assays: Aliquots of the PCA-precipitated incubations were neutralized with 50 ul of 1 M $KHCO_3$ and the glycogen was hydrolysed by amyloglucosidase (Sigma A 7255, from Rhizopus mold). Glucose levels in the hydrolysate and the initial incubate were determined enzymatically according to Bergmeyer (1974). Glucose production was estimated from the values obtained in the unhydrolysed samples, and glycogen content was calculated as the difference between the hydrolysate and the unhydrolysed sample.

ENZYME ASSAYS

Sea raven hepatocytes were prepared and incubated as previously described except that both 5 mM glucose and 5 mM serine were present in the final incubations. The incubates were centrifuged after the final 2 h incubation period at 1500 g for 45 sec., the supernatant discarded, and the cell pellet resuspended in a homogenization medium containing 20 mM HEPES, 1 mM dithiothreitol (DTT), and 20 mM NaF, at pH 7.6 (adjusted with NaOH). The samples were homogenized with a PCU-2 Polytron (Brinkman), and centrifuged at 11 000 g for 15 min (Sorvall RC-2B centrifuge) at 5°C. The supernatants were used for the

enzyme assays.

Enzyme activities were estimated by measuring the extinction of NADH at 340 nm with an LKB Ultrospec 4050 attached to a Zeiss chart recorder. Cuvette temperatures were maintained at 10°C (Haake refrigerated circulator). Phosphofructokinase and pyruvate kinase activities were estimated at both saturating (V_{opt} values) and sub-saturating concentrations of substrates (determined from substrate saturation curves). Control activities (without substrate addition) were subtracted from activities with substrate present. To obtain an idea of possible changes in the kinetic characteristics of the enzymes, both V_{opt} (activities at saturating substrate concentrations) and activity ratios (activity at subsaturating substrate concentrations against activity at saturating substrate concentrations) were calculated from values obtained with the crude homogenates. Activities are expressed as umoles.min^{-1}.(mg dry cell wt)$^{-1}$.

The specific enzyme conditions (from Walsh et al., 1985) were as follows:

Phosphofructokinase (PFK; EC 2.7.1.11). PFK was assayed in a medium containing 100 mM imidazole-HCl (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 0.15 mM NADH, and excess aldolase, triose phosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase. Activity was initiated by the addition of either 0.4 or 2.0 mM fructose-6-phosphate. Assays were also conducted in the

presence of fructose 2,6-bisphosphate (10 uM) at both substrate concentrations.

Pyruvate Kinase (PK; EC 2.7.1.40). PK was assayed in a medium containing 100 mM imidazole-HCl (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM ADP, 0.15 mM NADH, and excess PK-free LDH. Activity was initiated by the addition of either 2 mM or 10 mM phosphoenolpyruvate.

Phosphoenolpyruvate Carboxykinase (PEPCK; EC 4.1.1.32). PEPCK was assayed in a medium containing 100 mM imidazole-HCl (pH 7.6), 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.2 mM 2-deoxyguanosine 5'-diphosphate, 0.15 mM NADH, and excess MDH. Activity was initiated by the addition of 1 mM phosphoenolpyruvate.

DATA PRESENTATION AND STATISTICS

All experiments, with the exception of glycogen depletion rates, were performed in duplicate. All n-values listed on the tables and figures represent experiments on preparations from individual fish. All results are based on dry cell weight determined by drying cells for 48 h at 50°C. Due to dilution of the ^{14}C-glucose by the continuously liberated glycogen, absolute flux rates from glucose can not be determined. Glucose flux, therefore, is expressed as DPM converted.h^{-1}.(mg dry cell wt)$^{-1}$; as the actual radiotracer additions varied between experiments, the calculated values were normalized to the addition of 10^6 DPM. The rate of glycogen

depletion was dependent on the initial glycogen content, so statistics were performed on the calculated relative changes in the glycogen content. Ratio normality was established by the Kolmogorov-Smirnov test, and paired t-tests were performed on the ratios. Significance of correlation and regression coefficients were determined by t-tests (Kreyszig, 1970), and all other significant differences were determined by the paired Student t-test on the absolute values.

CHAPTER 3:

SEA RAVEN HEPATOCYTES AND GLUCAGON

INTRODUCTION

Glucagon is considered to effect hyperglycaemia in mammals (Turner and Bagnara, 1976). Similarly, glucagon induces hyperglycaemia when administered to intact teleosts (Thorpe and Ince, 1974; Murat and Serfaty, 1975; Ince and Thorpe, 1975,1977b; Carniero and Amaral, 1983), but it has inconsistent effects on liver glycogen (Plisetskaya, 1972; Chan and Woo, 1978; Castilla et al., 1979; Carniero and Amaral, 1983). *In vitro*, glucagon administration enhances gluconeogenesis from lactate and amino acids in trout hepatocytes, while increasing the apparent affinity of the glycolytic enzyme, pyruvate kinase, for its substrate phosphoenolpyruvate (Walton and Cowey, 1979a; Mommsen and Suarez, 1984). Similar changes in flux have been reported in liver slices of the toadfish (Tashima and Cahill, 1964). Glycogen levels are increased in trout liver slices (Morata et al., 1982), but reduced in isolated hepatocytes of the American eel (Renaud and Moon, 1980).

More comparative studies are required before a generalized role for glucagon in the teleost liver can be developed. In addition, glycogen effects need to be better defined as does the mechanistic basis for these glucagon

effects. The purpose of this study, therefore, was to establish the metabolic effects of glucagon on hepatocytes isolated from the sea raven, *Hemitripterus americanus*. The effects of glucagon on glycogen and glucose levels, alterations in glucose and amino acid fluxes, and selected enzyme activities are monitored in cells incubated with bovine glucagon.

RESULTS

General Characteristics of the Sea Raven Hepatocyte: The sea raven is a good organism for hepatocyte studies. This teleost is easy to maintain in the laboratory, feeds readily, and large numbers of intact hepatocytes are routinely obtained (Walsh et al., 1985). A 15 min. collagenase perfusion is adequate to liberate cells. The short exposure time to this protease may be of significance with respect to receptor sensitivity and hormone function. The preparation resulted in consistently greater than 95% cell viability as determined by the trypan blue exclusion technique, and red blood cell contamination was always less than 5%.

The rates of oxidation and of glucose production from lactate and seven selected amino acids by sea raven hepatocytes are shown on Table 3-1. Rates of oxidation are higher in each case than glucose production, with lactate being particularly low in this regard. Serine was the most

Table 3-1: Glucose and CO_2 production from selected substrates in hepatocytes isolated from the sea raven. Incubations proceeded at 10°C for 2 h in the final resuspension medium (see Materials and Methods). Rates are expressed as mean ± SE (range). n=3 fish. All substrates are present at 5 mM, with 3.0 to 4.5 x 10^5 DPM of the radioactive substrate. N.D., not detectable. P-alanine=phenylalanine.

	Rate of Production (umoles.h^{-1}.mg^{-1} dry wt.)	
	GLUCOSE	CO_2
Lactate	N.D.	7.8 ± 3.8 (3.8-15.5)
Alanine	0.26 (0-0.59)	7.2 ± 2.2 (5.4-11.7)
Serine	2.2 ± 0.9 (0.83-3.92)	5.2 ± 1.1 (3.4-7.2)
Glycine	0.3 ± 0.15 (0.14-0.42)	5.1 ± 2.1 (2.6-9.2)
Aspartate	0.2 ± 0.04 (0.15-0.32)	3.9 ± 1.0 (1.9-4.7)
P-alanine	0.19 ± 0.09 (0.02-0.29)	7.9 ± 1.7 (5.3-11.3)
Leucine	N.D.	2.8 ± 1.2 (1.1-5.1)
Histidine	N.D.	0.4 (0-0.7)

readily utilized substrate and was, therefore, used in all further studies. Gluconeogenic rates were on the low side of the range reported in other teleost studies except for lactate which was very low (e.g. Walton and Cowey, 1979a; Renaud and Moon, 1980; French et al., 1981; Mommsen and Suarez, 1984; Moon et al., 1985).

Metabolite Profiles: Sea raven hepatocytes show a steady depletion of glycogen throughout the incubation period (Fig. 3-1). The rate of this loss was related to the initial glycogen content such that greater lability occurred at high initial concentrations (Fig. 3-2). These observations are similar to those in salmon and trout hepatocytes (Mommsen, personal communication). The depletion rates ranged from 0 to 15% h^{-1} with a mean of approximately 4% h^{-1}. Glucagon administration resulted in greater glycogen loss, although the only significant difference occurred at 1.5 h in the presence of serine (Fig. 3-1).

Hepatocytes incubated with either substrate significantly increased total glucose content (Fig. 3-3). Over the two hour period, glucose levels with no hormone present increased to a greater extent in the presence of 5 mM glucose (0.014 ± 0.003 mmoles.g^{-1}) than in the presence of 5 mM serine (0.011 ± 0.004 mmoles.g^{-1}) ($p<0.05$). Glucagon addition at 10^{-8} M significantly

Fig. 3-1: Glycogen content of sea raven hepatocytes as affected by bovine glucagon. Hepatocytes were incubated in the final resuspension medium containing either 5 mM glucose (solid symbols) or 5 mM serine (open symbols). No hormone (circles); 10^{-8} M glucagon (triangles). Rates of depletion dependent on the initial glycogen content (T_0 of the pre-incubation, see Fig. 3-2), thus statistics were performed on the normally distributed (Kolmogorov-Smirnoff test) ratios of relative glycogen changes within hepatocytes. Significant differences were determined by the paired t-test. n=5 at all points. * $p<0.05$, against control; ** $p<0.05$, against 0 h.

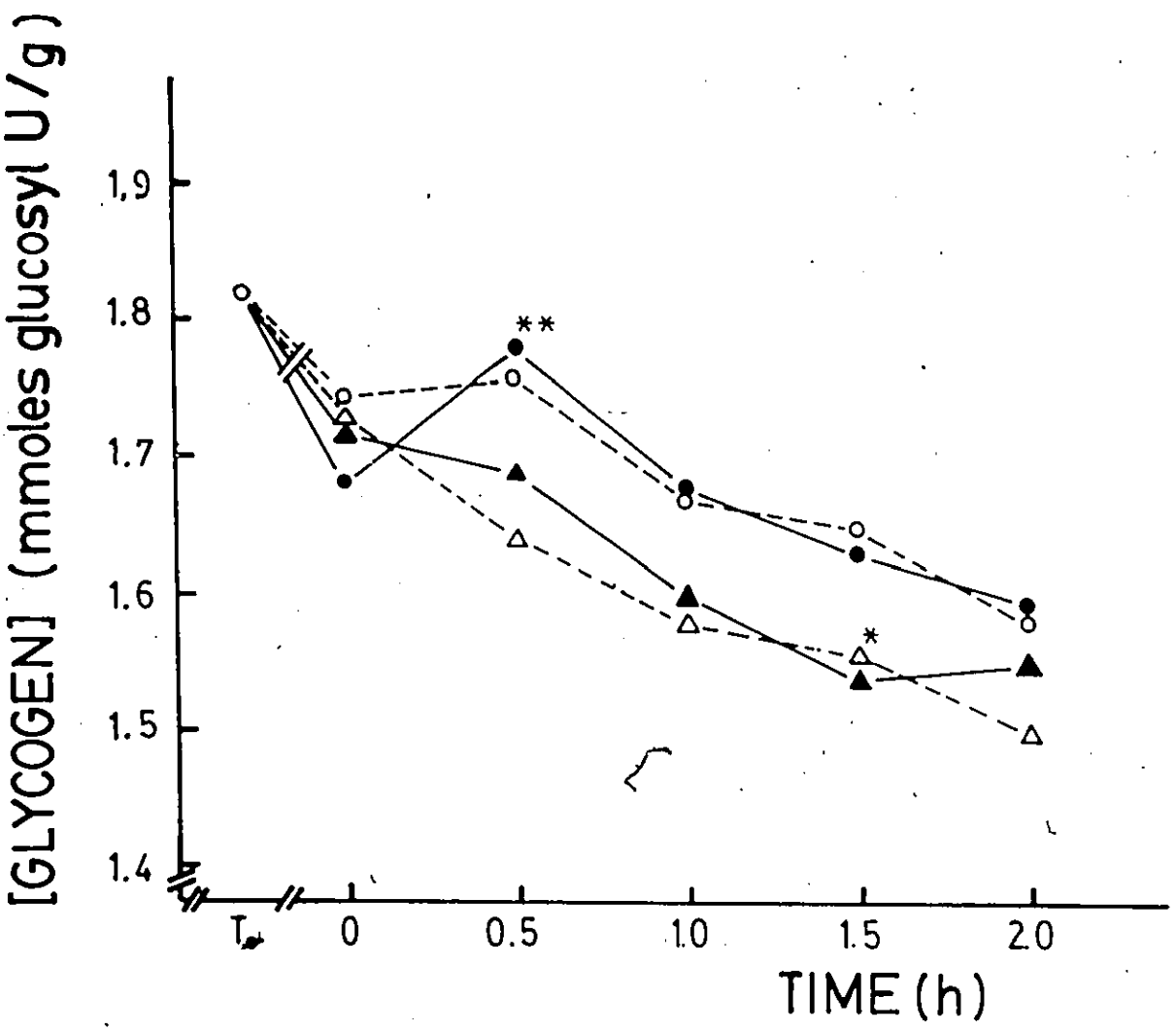

[GLYCOGEN] (mmoles glucosyl U/g)
1.9
1.8
1.7
1.6
1.5
1.4
**
*
T
0
0.5
1.0
1.5
2.0
TIME (h)

Fig. 3-2: The dependence of glycogen depletion rates on the initial glycogen content (mmoles glucosyl units.g^{-1}) of the sea raven hepatocytes. Initial samples (T_0) were taken following the cell isolation, and the final samples (t_f) were collected after the final 2 h incubation (no hormone). Significance of the correlation and regression coefficients were determined by t-tests at $p<0.05$.

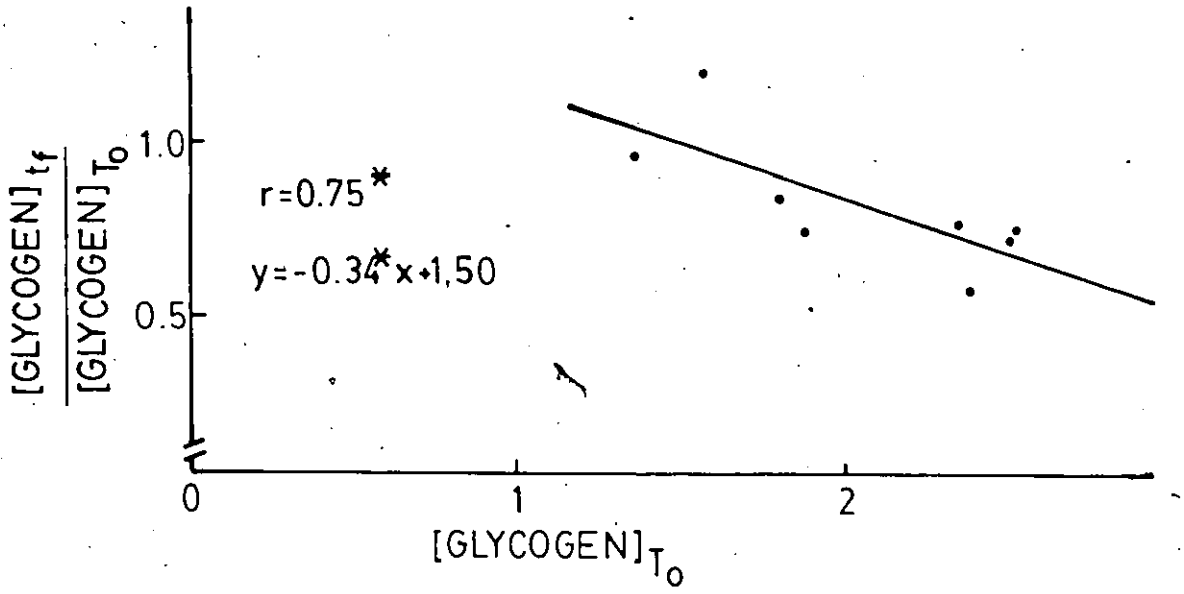
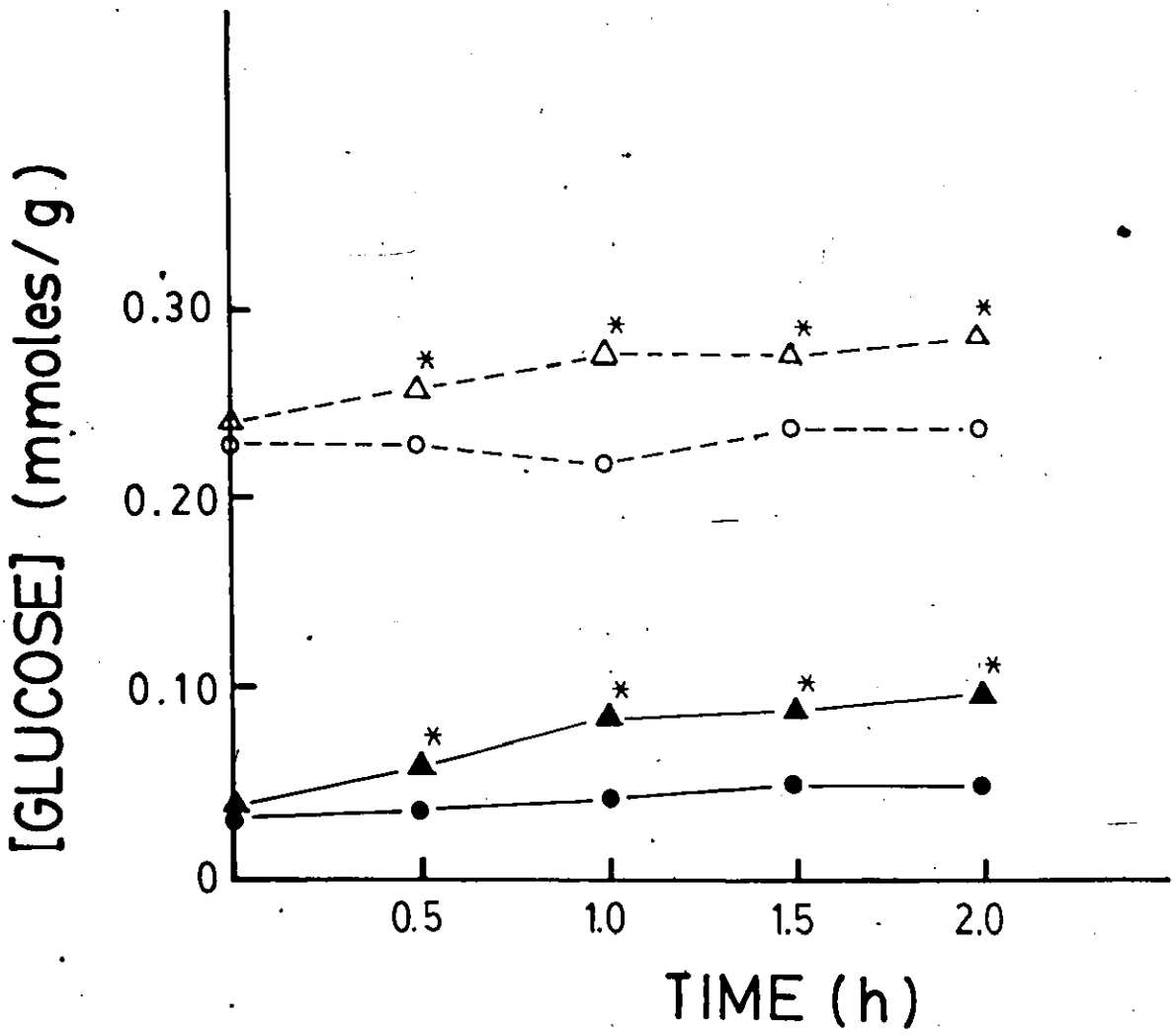

[GLYCOGEN]tf / [GLYCOGEN]To
1.0
0.5
r=0.75*
y=-0.34*x+1,50
0
1
2
[GLYCOGEN]To

Fig. 3-3: Total glucose levels (medium + cells) in sea raven hepatocytes incubated with and without bovine glucagon. Fish and experimental protocol as in Fig. 3-1. 5 mM glucose (open symbols); 5 mM serine (closed symbols). No hormone (circles); 10^{-8} M glucagon (triangles). * $p<0.05$, against 0 h; n=5.


[GLUCOSE] (mmoles/g)
0.30
0.20
0.10
0
0.5
1.0
1.5
2.0
TIME (h)

increased glucose production with increases of 0.030 ± 0.009 mmoles.g^{-1} (5 mM glucose) and 0.041 ± 0.016 mmoles.g^{-1} (5 mM serine) at 2 h (no significant difference between substrate effects in the presence of glucagon). The hormone also increased the ratio of glucose production (Fig.3-3) to glycogen loss (Fig. 3-1) over the 2 h incubation from 20% to 29% ($p<0.05$) in the presence of glucose, suggesting increased flow of glucosyl units from glycogen to glucose.

Flux Rates: The incorporation of radioactive glucose carbon into glycogen, CO_2, and protein is shown in Table 3-2. As glycogen is continuously breaking down to dilute the radioactive glucose, rates are presented as DPM-glucose incorporated.h^{-1}.mg^{-1}. dry wt. (corrected to the addition of 10^6 DPM). No radioactivity was detected in lipid or lactate in sea raven hepatocytes incubated with glucose, while CO_2 was the favoured endproduct. A relatively small amount of radioactivity was localized to protein, and approximately twice as much was incorporated into glycogen. A small decrease in the glucose flux to CO_2 was noted in the presence of glucagon. This may be attributed to an increased dilution of the isotope as glycogen breakdown was enhanced (Fig. 3-1). However, the flux of glucose to glycogen actually increased slightly, suggesting the possibility of a direct effect of glucagon

Table 3-2: The incorporation of [U-^{14}C]-glucose into endproducts of sea raven hepatocytes incubated with various concentrations of bovine glucagon. Cells were pre-incubated for 3 h with or without glucagon before a 2 h incubation with added radioactive glucose. Values are expressed as mean (SE). Flux was corrected to the addition of 10^6 DPM. Significant differences determined by the paired t-test; n=5. N.A., not assayed.

	Rate of Incorporation (DPM.h^{-1}.mg^{-1} dry wt.)		
	GLYCOGEN	CO_2	PROTEIN
No Hormone	71.5 (21.3)	11 600 (2 400)	41.4 (10.2)
10^{-9} M Glucagon	73.2 (21.7)	9 900 (2 400)	40.5 (12.0)
10^{-8} M Glucagon	78.0 (21.5)*	9 300 (3 100)	45.3 (12.9)
10^{-5} M Glucagon	N.A.	8 800 (2 500)	N.A.

Fig.3-4: Glycogen, glucose, and CO_2 production from serine, and serine incorporation into protein as affected by selected concentrations of glucagon (noted on the abscissa) in sea raven hepatocytes. Experiments were conducted as stated on Table 3-2. Hormone molarity shown below the bars. All experiments were paired, therefore SE is presented only around the control values. The experiments were performed in late summer. Significant differences were determined by paired t-tests; n=5. * $p<0.05$, against No Hormone.

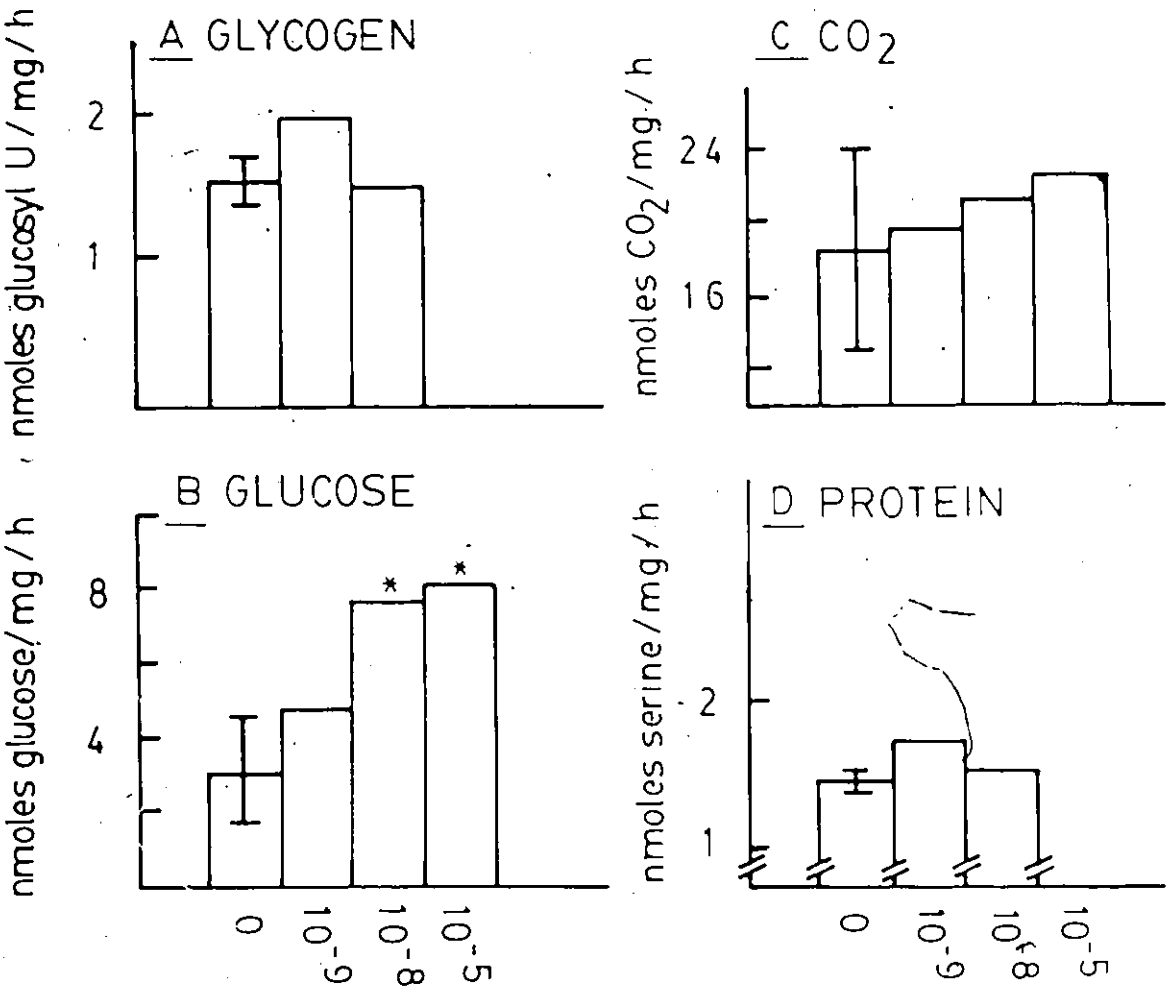
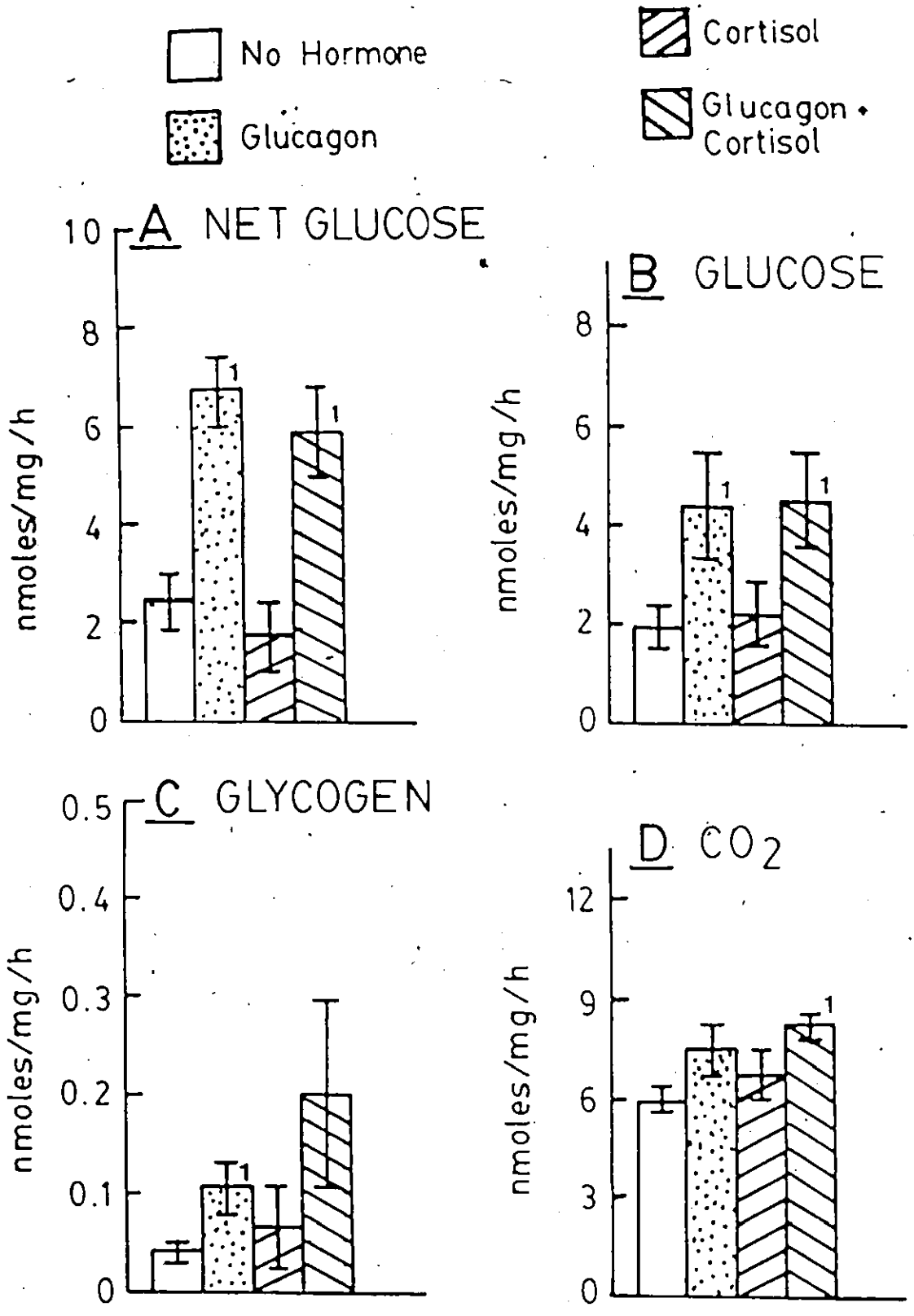

A GLYCOGEN
nmoles glucosyl U / mg / h
2
1
C CO_2
nmoles CO_2 / mg / h
24
16
B GLUCOSE
nmoles glucose / mg / h
8
4
*
*
0
10^{-9}
10^{-8}
10^{-5}
D PROTEIN
nmoles serine / mg / h
2
1
0
10^{-9}
10^{-8}
10^{-5}

Fig. 3-5: Glucose production (no radioactive addition) (A, n=7), and serine incorporation into glucose (B, n=7), glycogen (C, n=6) and CO_2 (D, n=7) as affected by bovine glucagon (10^{-7} M), cortisol (10^{-7} M) and glucagon + cortisol (10^{-7} M each). The experiments were conducted as stated on Table 3-2, during the spring. Significant differences were determined by paired t-tests at $p<0.05$. 1) different from no hormone.


No Hormone
Glucagon
Cortisol
Glucagon + Cortisol
A NET GLUCOSE
B GLUCOSE
C GLYCOGEN
D CO_2
nmoles/mg/h
nmoles/mg/h
nmoles/mg/h
nmoles/mg/h
0
2
4
6
8
10
0
2
4
6
8
0
0.1
0.2
0.3
0.4
0.5
0
3
6
9
12
1
1
1
1
1

at this step. No effect on the glucose flux to protein was found, nor did the hormone alter flux to lactate or lipid.

Serine flux is presented as either endproduct incorporation (glucose, CO_2, and glycogen) or substrate incorporation into endproducts (protein) (Fig. 3-4 and 3-5). An initial experiment determined the effects of selected glucagon concentrations (Fig. 3-4; performed in late summer), and a second experiment determined the interactive effects of glucagon and cortisol (Fig. 3-5; performed in the spring). While the control rates in the spring experiments were lower than the autumn experiment, CO_2 production (oxidation) remained six times greater than glucose production in both cases. Glycogen production was approximately half that of glucose production in the late summer study, but the flux to glycogen in the spring was only 2% of that found in the late summer, suggesting seasonal differences particularly in glycogen metabolism. The sole effect of glucagon in the late summer experiment was to increase serine flux to glucose by 2.5 times (Fig. 3-4). Flux to glycogen and CO_2 were also stimulated in the spring experiment (Fig. 3-5), demonstrating seasonal differences in liver metabolism. The addition of both glucagon and cortisol slightly increased serine flux to CO_2 above the rates with glucagon alone (Fig. 3-5). Cortisol (10^{-7} M) alone did not influence any of the parameters studied.

Table 3-3: Optimal activities (V_{opt}; umoles.min^{-1}.g^{-1} dry wt.) and activity ratios (enzyme activities at low to saturating substrate concentrations) in crude homogenates of sea raven hepatocytes as affected by glucagon. Cells were pre-incubated for 3 h with or without glucagon before a final 2 h incubation. Enzyme assays were performed following the final incubation. Values are mean (SE). Significant differences determined by paired t-tests. n=8, 10^{-8} M glucagon (except PFK Act. Ratio, n=6); n=5, 10^{-5} M glucagon. * $p<0.05$; ** $p<0.075$.

		GLUCAGON, 10^{-8} M		GLUCAGON, 10^{-5} M	
		control	hormone	control	hormone
PFK	V_{opt}	0.62 (0.08)	0.48 (0.09)*	0.61 (0.13)	0.42 (0.10)
	Act. Ratio	0.13 (0.04)	0.13 (0.04)	0.15 (0.05)	0.10 (0.04)
PK	V_{opt}	1.48 (0.10)	1.29 (0.13)**	1.42 (0.08)	1.18 (0.09)
	Act. Ratio	0.31 (0.02)	0.30 (0.01)	0.29 (0.02)	0.29 (0.03)
PEPCK	V_{opt}	0.13 (0.01)	0.15 (0.01)	0.13 (0.01)	0.18 (0.02)*

Enzymes: Consistent with increased glucose production (Fig. 3-2), the characteristics of PFK, PK, and PEPCK changed (Table 3-3). Glucagon decreased the optimal activities of the glycolytic enzymes, PFK and PK (PK significant at $p<0.075$) without affecting the activity ratios (activity at low substrate to activity at high substrate concentrations). This response was more consistent at the lowest glucagon concentration used. The activity of the gluconeogenic enzyme, PEPCK, was significantly stimulated by glucagon at 10^{-5} M.

DISCUSSION

The hepatocytes of the sea raven are capable of utilizing a variety of amino acid substrates (Table 3-1). Glucose production from lactate and glucose flux to lactate were undetectable. These results are similar to those previously reported for sea ravens by Walsh et al. (1985), although the lactate oxidation rates reported here are higher. The large difference between the rates of lactate gluconeogenesis and oxidation requires more study. It is known that sea ravens maintain low LDH titres in the liver (Walsh et al., 1985) and may not release lactate from the skeletal muscle, even after exhaustive exercise (Milligan and Farrell, 1985). Whether these are related to the low

rate of gluconeogenesis is unknown. Lactate production accounts for 80-90% of the total glycolytic flux in rat hepatocytes (Kimmig et al., 1983), whereas glucose was converted to CO_2 in these sea raven hepatocyte experiments (Table 3-2). The lack of lactate formation from glucose and the small fraction of added glucose converted to endproducts studied (about 1%), suggests this tissue has a low potential to utilize added glucose. Up to 75% of the utilized serine is converted to glucose and glycogen (assuming a 2:1 ratio of serine conversion to carbohydrates (Phillips and Hird, 1977)), and therefore the tissue may be directed primarily towards glucose synthesis from amino acids. While most metabolic pathways exhibit low activities, including lipid oxidation (Walsh et al., 1984), gluconeogenic rates are comparable, although slightly lower, to other teleost species studied to date (see e.g. Moon et al., 1985). With the exception of lactate, rates are similar to those found in eel hepatocytes (Renaud and Moon, 1980) and in trout hepatocytes, where serine was also the most readily utilized substrate (Walton and Cowey, 1979a; French et al., 1981; Mommsen and Suarez, 1984).

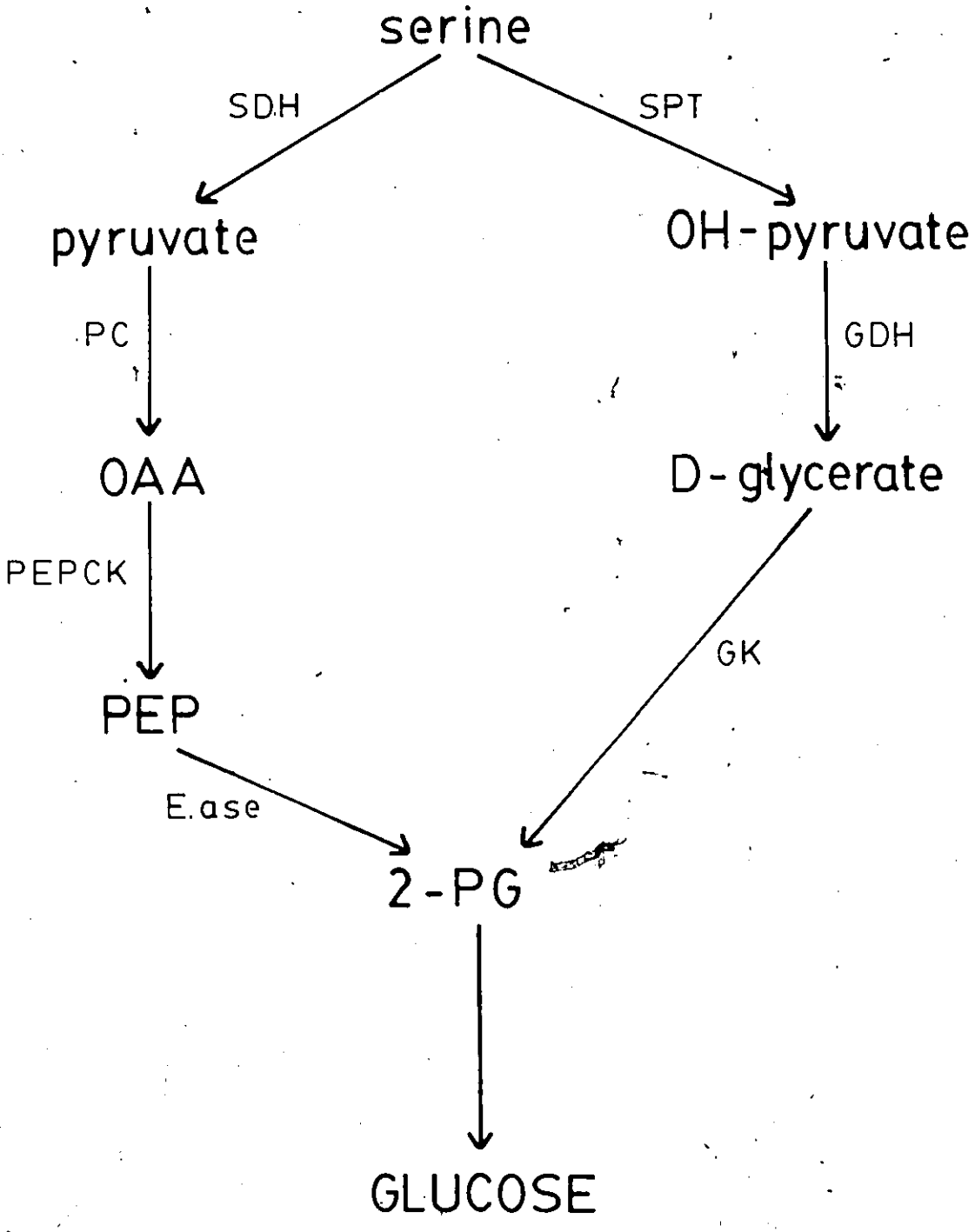
Glucagon addition to sea raven hepatocytes enhanced glycogenolysis above controls (Fig. 3-1). An increase of 50% in the ratio of glucose production to glycogen breakdown (from 20% to 29%) demonstrates that the liberated glucosyl units from glycogen account for some of the enhanced glucose production. Also, the enhanced glucose

production from serine (Fig. 3-4 and 3-5) indicates that glucagon stimulates gluconeogenesis from non-carbohydrate precursors. Thus, as in the rat (Hems and Whitton, 1980; Kraus-Friedmann, 1984) and consistent with changes in teleost liver phosphorylase (Umminger and Benziger, 1975; Umminger et al., 1975; Vernier and Sire, 1978) and gluconeogenesis (Tashima and Cahill, 1964; Walton and Cowey, 1979a; Mommsen and Suarez, 1984), glucagon-enhanced glucose production is accomplished by this dual mechanism. Studies by Morata et al.(1982) on trout liver slices found glycogen levels increased with glucagon and cAMP, suggesting glucose release originated primarily from gluconeogenesis. Glucagon had little effect on glucose utilization, with one notable exception (Table 3-2). It caused a slight increase in glucose flux to glycogen, an observation inconsistent with the apparent role of the hormone in this tissue. Tashima and Cahill (1964) reported a similar effect in toadfish liver slices.

In the rat liver the effects of glucagon on gluconeogenesis are enhanced by the permissive role of glucocorticoids. This is a consequence of the enhanced stimulation of the enzyme PEPCK (Kletzein et al., 1981). Although no effects on serine gluconeogenesis were found in sea raven hepatocytes incubated with glucagon and cortisol (Fig. 3-5) coordinate regulation of PEPCK cannot be ruled out. This appears unlikely as in rainbow trout liver, and other carnivorous vertebrates, serine is metabolized

Fig. 3-6: Pathways of serine gluconeogenesis in trout (from Walton and Cowey, 1979b). SDH, serine dehydratase; PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; E.ase, enolase; SPT, serine-pyruvate transaminase; GDH, glycerate dehydrogenase; GK, glycerate kinase.

SERINE METABOLIZING PATHWAYS



through a pathway which bypasses PEPCK as shown on Fig. 3-6 (Walton and Cowey, 1979b). Rather than the carbon moving through pyruvate, serine is metabolized to hydroxypyruvate by serine-pyruvate transaminase (SPT), and two further reactions result in the formation of 2-phosphoglycerate. From this point serine gluconeogenesis occurs through the normal gluconeogenic pathway, including the regulatory enzymes, PFK and FBPase. If this is the case in the liver of the sea raven, serine gluconeogenesis would be unaffected by changes in PEPCK. Further studies of cortisol and glucagon effects on PEPCK activities and flux from other substrates (e.g., alanine) would elucidate this mechanism. Mommsen and Suarez (1984) have reported a greater enhancement of alanine than lactate gluconeogenesis with glucagon in rainbow trout hepatocytes, implying specific effects of the hormone on precursor conversion. The addition of both hormones to sea raven hepatocytes, however, significantly increased serine oxidation above their individual additions (Fig. 3-5). Cortisol and glucagon may be affecting oxidative pathways and amino acid uptake, respectively.

The amplification of the rates of serine flux to glycogen and CO_2 in the presence of glucagon that is apparent in the spring experiments (Fig. 3-5) compared to the late summer experiment (Fig. 3-4) may be due to the depressed control rates in the spring experiment (Fig.

3-5). The limiting factor in the spring controls may be substrate availability and/or uptake. A consistent glucose to CO_2 flux ratio of six in both experiments is suggestive of a single mechanism causing the depressed activities in the spring experiments.

The enzyme changes observed with glucagon addition (Table 3-3) are consistent with the altered state of the hepatocytes, but unlike those previously reported. Mommsen and Suarez (1984) have shown a glucagon-induced increase in the K_m for phosphoenolpyruvate of trout hepatocyte PK following a 1 h incubation with 10 uM glucagon or 100 uM cAMP; there was no change in activities at high PEP concentrations, only low. These data support a mechanism involving the phosphorylation of trout PK by a cAMP-dependent protein kinase as in the rat liver (Riou et al., 1978). In contrast, after the 5 h incubation of the present study, PK activities increased, without a change in the activity ratio, implying no change in the K_m(PEP) (Table 3-3). These differential effects may be related to the duration of the two experiments. Feliu et al.(1976) reported that an increase in the K_m(PEP) for PK from isolated rat hepatocytes occurred within 5 min of glucagon addition, with no change in the V_{max}. Taunton et al.(1974) also showed that short-term changes did not involve changes in protein synthesis, but suggested that long-term effects may involve changes in the rate of specific enzyme synthesis and thus, maximal (optimal)

activities. As glucagon increased the V_{opt} of PFK as well, the same argument could apply to this enzyme (Table 3-3), which are at variance to the short-term changes in the rat hepatocytes (Pilkis et al., 1979). The increased activity of PEPCK, measured under saturating substrate conditions, is consistent with enhanced gluconeogenesis and the results obtained with rat hepatocytes (Iynedjian et al., 1985). The enzyme changes noted in this study are consistent with enhanced gluconeogenesis, but Groen et al. (1983) suggest that PC is the key regulating enzyme in gluconeogenesis, thus future studies of gluconeogenesis should consider the role of this enzyme. The lack of any observed changes in the activity ratios (i.e., phosphorylation-dephosphorylation) may be due to the composition of the homogenization medium (Pilkis et al., 1979).

In summary, the role of glucagon in sea raven hepatocytes is to increase hepatic glucose production by enhancing both gluconeogenesis and glycogenolysis. Changes in the enzymes assayed are consistent with the enhanced gluconeogenesis. Thus, glucagon is directly involved in the regulation of glucose levels in a manner resembling that seen in the mammalian liver and other species where it has been investigated. Whether the effects on PK are distinct requires further studies of the time course of the response.

CHAPTER 4:

SEA RAVEN HEPATOCYTES AND INSULIN

INTRODUCTION

Insulin has a well-established hypoglycaemic action in the intact teleost (Thorpe, 1976), and radioisotope studies and metabolite measurements have established a general anabolic role for insulin in teleost muscle (Castilla and Murat, 1975; Ince and Thorpe, 1976; Ablett et al., 1981b). Insulin effects on liver metabolism, however, are still poorly defined, particularly with respect to glycogen metabolism (Inui and Yokote, 1975; Ince and Thorpe, 1976; Ablett et al., 1981a; Carniero and Amaral, 1983). Intact animal studies have major drawbacks (see Chap. 1), and recently, isolated hepatic studies have led to a new understanding of insulin actions. Results with preparations, including isolated hepatocytes, liver slices, and perfused livers, support a general anabolic role for insulin, with enhanced glycogen content and amino acid uptake and utilization resulting from insulin administration (Inui and Ishioka, 1983; Plisetskaya et al., 1984; Ottolenghi et al., 1985). Hepatocyte preparations have become the preferred tool for liver studies in teleosts (Moon et al., 1985). Preparations of salmon hepatocytes are sensitive to presumably physiological doses of insulin (Plisetskaya et al., 1984), whereas *in*

vivo studies (Ince and Thorpe, 1974; Lewander et al., 1976; Carniero and Amaral, 1983) and liver perfusions and slice (de Vlaming and Pardo, 1975; Ottolenghi et al., 1985) preparations require relatively high hormone doses to obtain a response.

Insulin functions to increase glucose utilization in the rat liver by increasing glucose conversion to lipid, increasing glycogen synthesis and glycolysis (Turner and Bagnara, 1976), and depressing the glucagon-enhanced gluconeogenic flux (Hue, 1982). The teleost liver is biochemically different from that of mammals, and there is no a priori reason for the mechanism of insulin action to be the same. For example, fish livers examined to date exhibit poor glucose utilization, have only a low K_m hexokinase (Ureta, 1981), and are considered poor "glucostats" (Cowey and Sargent, 1979). Hepatectomy is not acutely lethal in some fish, including an Anguillid species (Inui, 1969).

This chapter attempts to establish the effects of insulin on the metabolism of hepatocytes isolated from the sea raven. Key enzyme activities and metabolite fluxes are examined in the presence of porcine and teleost insulins, and the interactive effects of teleost insulin and glucagon are also determined. Teleost (swordfish) and mammalian (porcine) insulins are used to compare their effects, as the potency of species-specific insulins has been questioned (Ince and Thorpe, 1974; Leibson et al., 1976;

Plisetskaya et al., 1984).

RESULTS

Metabolite Profiles: Mammalian (porcine) insulin at 10^{-9} M maintained the glycogen content of sea raven hepatocytes for 1.5 h, in both the presence of 5 mM glucose or 5 mM serine (Fig. 4-1); this result is especially marked with serine. In contrast, glycogen content dereased in the presence of swordfish insulin at 10^{-8} M, in a manner similar to the control (no hormone) incubations (Fig. 4-1). Differential effects were also noted with respect to total glucose content (Fig. 4-2). Glucose content with porcine insulin paralleled the control (no hormone) condition, but swordfish insulin caused a pronounced increase in content. Consistent with these changes, the ratio of net glucose production (Fig. 4-2) to glycogen breakdown (Fig. 4-1) over the 2 h incubation with glucose present decreased from 22% to 9% with porcine insulin, while with swordfish insulin the ratio increased from 19% to 69%.

Flux Rates: Table 4-1 indicates radioactive glucose carbon incorporated into various endproducts. Control (no hormone) flux to glycogen was the same in both insulin experiments (porcine insulin, late summer; swordfish insulin, autumn) while protein rates were lower, and oxidation rates were

Fig. 4-1: Changes in glycogen content in hepatocytes isolated from the sea raven in the presence of porcine and swordfish insulins. Experiments were performed in the autumn. Cells were pre-incubated for 3 h, and resuspended at 0 h in fresh FRM containing either 5 mM glucose (A) or 5 mM serine (B). No hormone (circles); 10^{-9} M porcine insulin (closed triangles); 10^{-8} M swordfish insulin (open triangles). Significant differences were determined by paired t-tests on the normally distibuted (Kolmogorov-Smirnoff test) ratios of glycogen contents between T_0 and 2 h; n=5 at all points. * $p<0.05$, against no hormone; ** $p<0.075$, against no hormone; *** $p<0.05$, against 0 h.

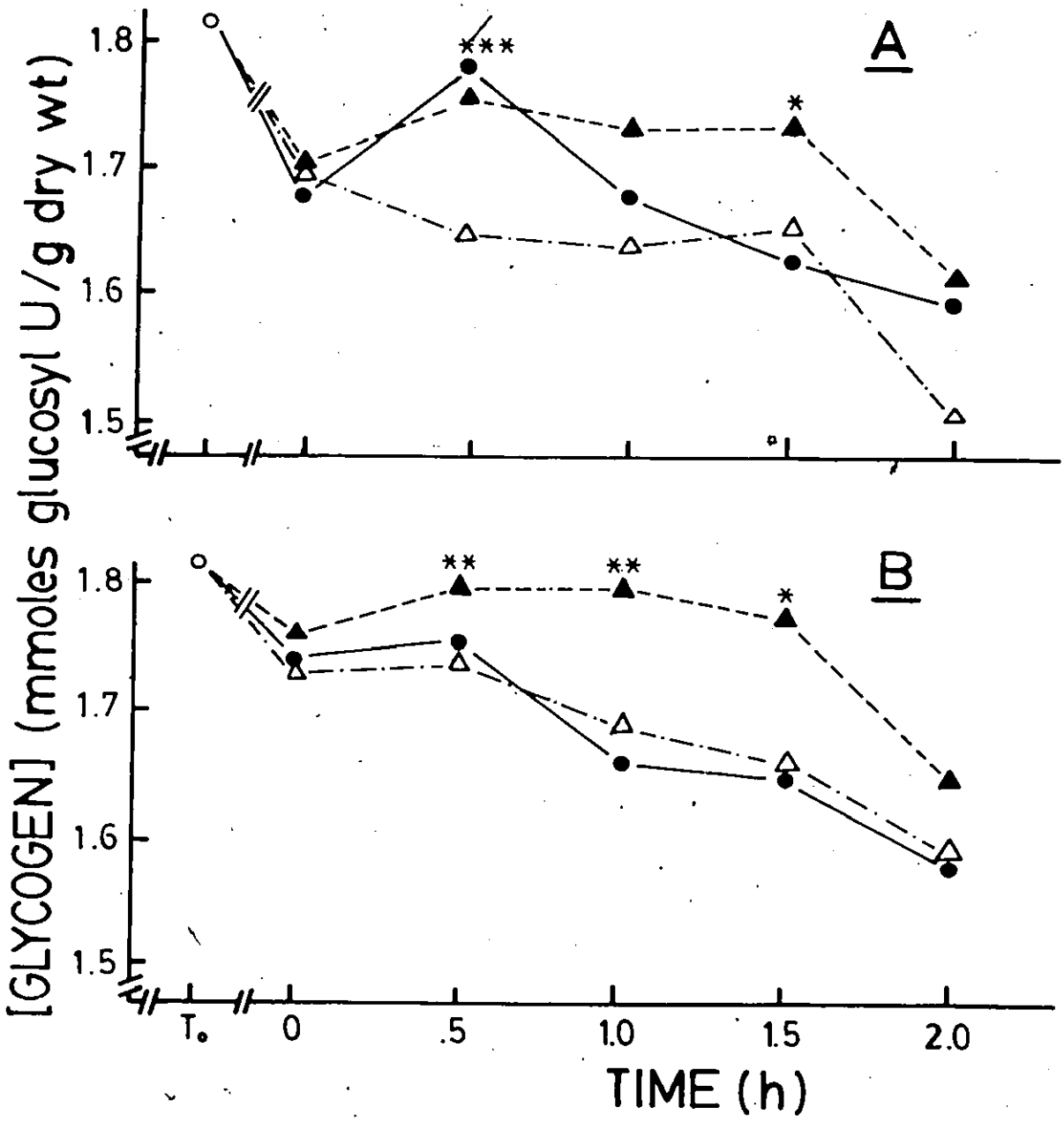

A
B
[GLYCOGEN] (mmoles glucosyl U/g dry wt)
1.8
1.7
1.6
1.5
***
*
**
**
*
T₀
0
.5
1.0
1.5
2.0
TIME (h)

Fig. 4-2: Total glucose content in hepatocytes prepared from the sea raven and incubated with porcine and swordfish insulins. Fish and experimental protocol as in Fig. 4-1. (A) 5 mM glucose; (B) 5 mM serine. No hormone (circles); 10^{-9} M porcine insulin (solid triangles); 10^{-8} M swordfish insulin (open triangles). Significant differences were determined by paired t-tests on the absolute values. n=5 at all points. * $p<0.05$, against no hormone; ** $p<0.075$, against no hormone.

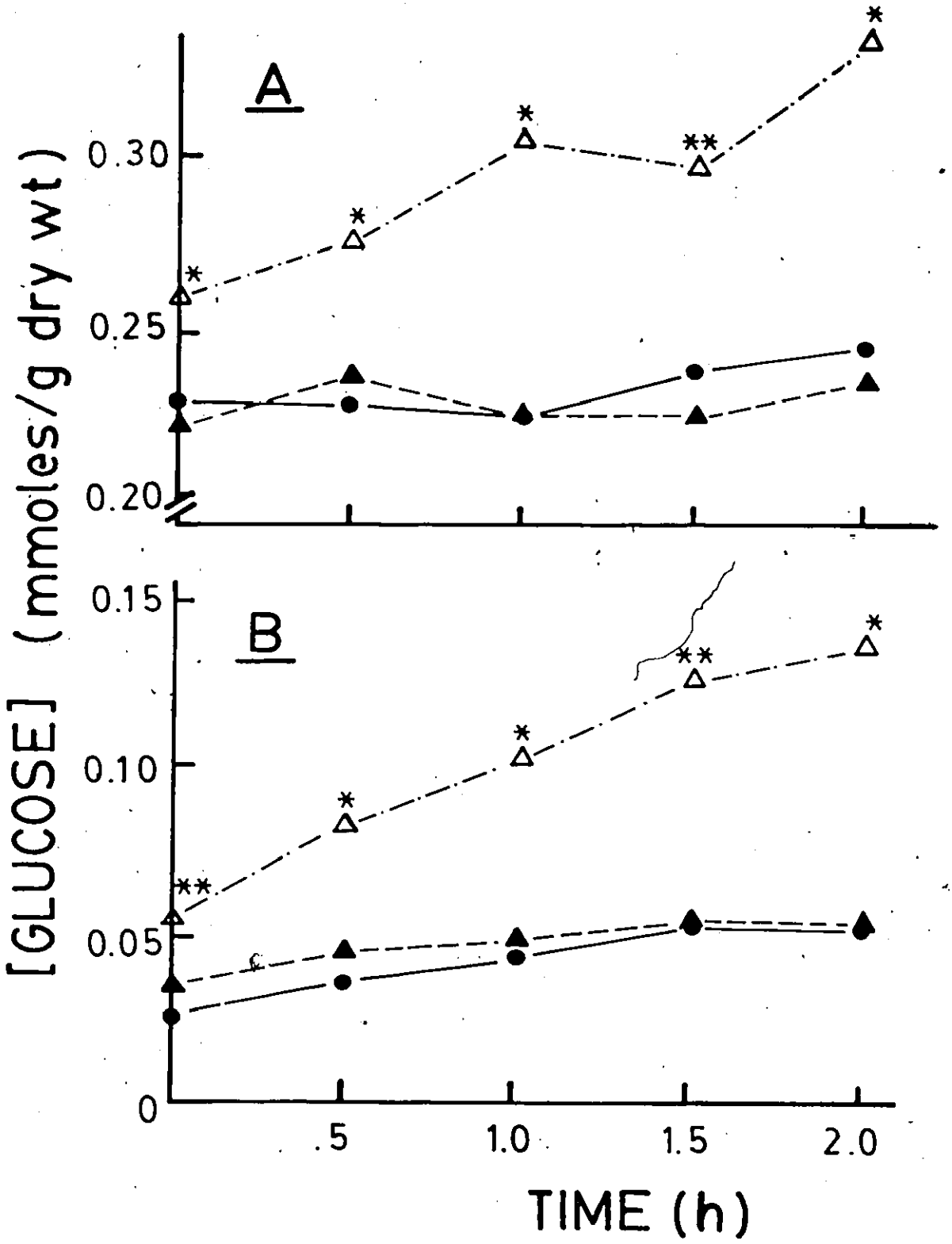

A
B
0.30
0.25
0.20
0.15
0.10
0.05
0
[GLUCOSE] (mmoles/g dry wt)
.5
1.0
1.5
2.0
TIME (h)

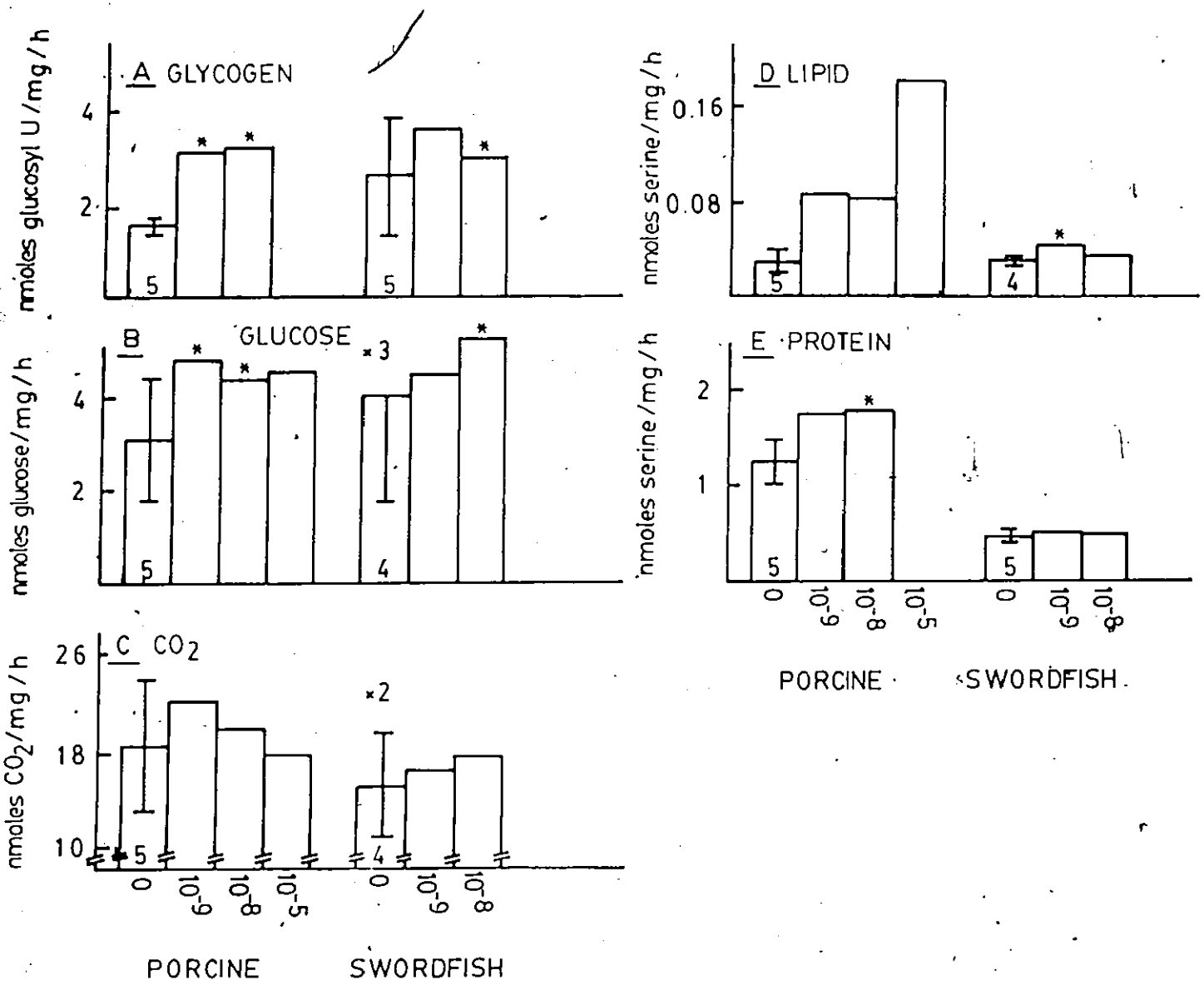
TABLE 4-1: The incorporation of [U-^{14}C]-glucose into endproducts of sea raven hepatocytes incubated with various concentrations of porcine and swordfish insulins. The experiments were performed in late summer (porcine insulin) and autumn (swordfish insulin). Cells were pre-incubated 3 h with or without insulin before a 2 h incubation with added radioactive glucose. Values are as mean ± SE, adjusted to 10^6 DPM per incubate. Significant differences were determined by paired t-tests; n=5. N.A. Not assayed.

HORMONE	CONDITION	GLYCOGEN	CO_2	PROTEIN
		n=5	n=5	n=5
Porcine Insulin	No Hormone	71.5 ± 21.3	11 600 ± 2 426	39.0 ± 13.0
	10^{-9} M	79.8 ± 22.0*	14 940 ± 4 471	50.0 ± 10.0
	10^{-8} M	78.0 ± 21.5	14 380 ± 4 363	55.0 ± 10.0
	10^{-5} M	N.A.	12 460 ± 3 601	N.A.
		n=4	n=5	n=4
Swordfish Insulin	No Hormone	70.6 ± 3.9	22 280 ± 4 645	20.0 ± 3.2
	10^{-9} M	77.0 ± 8.6	23 240 ± 5 275	22.8 ± 4.6
	10^{-8} M	79.5 ± 7.7	19 340 ± 4 177	23.4 ± 4.9

higher in the autumn experiments. Porcine insulin significantly increased isotope incorporation into glycogen. However, this does not neccesarily indicate increased glucose flux to glycogen as the hormone also decreased glycogen depletion (Fig. 4-1), which could decrease the dilution of the radioactivity and result in an "apparent" increase in flux. No significant hormone effects were noted on glucose oxidation or incorporation into protein, although the trend is to increase incorporation. As in the previous experiments (Chap. 3), oxidation was the principal route of glucose metabolism in these cells. No consistent hormone effects on oxidation are noted.

Control rates of serine oxidation in the experiments with swordfish insulin (autumn experiments) were higher than when porcine insulin was used (late summer), while flux to protein was lower in the former experiment, and flux to glycogen and to lipid at both times were comparable (Fig. 4-3). These seasonal differences are similar to those found for glucose utilization (Table 4-1). Serine oxidation was unaffected by mammalian insulin, while incorporation into lipid tends to increase, but not significantly (Fig. 4-3). Serine incorporation into protein was enhanced. Effects on carbohydrates were generally apparent at 10^{-9} M mammalian insulin, while effects on protein appeared only at 10^{-8} M, changes equivalent with those found in salmon hepatocytes (Plisetskaya et al.,

Fig. 4-3: Serine incorporation into glycogen, protein, and lipid and glucose and CO_2 production from serine as affected by porcine and swordfish insulins in hepatocytes isolated from the sea raven. Experimental protocol as in Fig. 4-1. Hormone molarity stated below the bars and the n-value is within the bars. Vertical bars around the control values represent SE. The experiments were performed in late summer (porcine insulin) and autumn (swordfish insulin). Multiplication factors (B and C) denote the multiplication required to obtain the absolute rates with the swordfish insulin. Significant differences determined by paired t-tests. (A) glycogen production; (B) glucose production; (C) CO_2 production; (D) lipid and (E) protein incorporation. * $p<0.05$, against no hormone.


A GLYCOGEN
nmoles glucosyl U/mg/h
B GLUCOSE
nmoles glucose/mg/h
C CO2
nmoles CO2/mg/h
D LIPID
nmoles serine/mg/h
E PROTEIN
nmoles serine/mg/h
PORCINE
SWORDFISH

1984) and consistent with the dose differences of metabolic and growth-promoting aspects of rat epididymal cells (King and Kahn, 1981).

Swordfish insulin had effects similar to mammalian insulin with respect to carbohydrate metabolism and oxidation in the autumn experiments (Fig. 4-3). The small increase in flux to lipid was significant, but no effect on protein metabolism was noted. Control flux to protein was lower in the fish compared to the mammalian insulin studies, and as these animals were used during active spawning (autumn), the lack of the effect may be linked to seasonality,

In the rat liven insulin generally suppresses glucagon-stimulated gluconeogenesis (Hue, 1982). The addition of swordfish insulin to sea raven hepatocytes did not offset the glucagon effect on net glucose production (Fig. 4-4) or serine flux to glucose (Fig. 4-5). In fact, the two hormones had additive effects on glucose production and gluconeogenesis. The two hormones were, however, antagonistic with respect to serine flux to glycogen and CO_2 (Fig. 4-5).

Enzymes: Fructose 2,6-bisphosphate (F2,6-P) has been found to modulate mammalian liver PFK and FBPase activities (Hers et al., 1982). In the sea raven hepatocyte, F2,6-P increased both the maximal activities of PFK and the

Fig. 4-4: Glucose production in sea raven hepatocytes incubated with bovine glucagon (10^{-7} M), swordfish insulin (10^{-8} M) and insulin + glucagon (10^{-7} M and 10^{-8} M, respectively). The experimental protocol is noted on Fig. 4-1; no radioactive substrate was involved. The experiments were performed in the spring. Significant differences determined by paired t-tests at $p<0.05$. 1) different from control; 2) different from glucagon; 3) different from insulin. Shading as in Fig. 4-5.

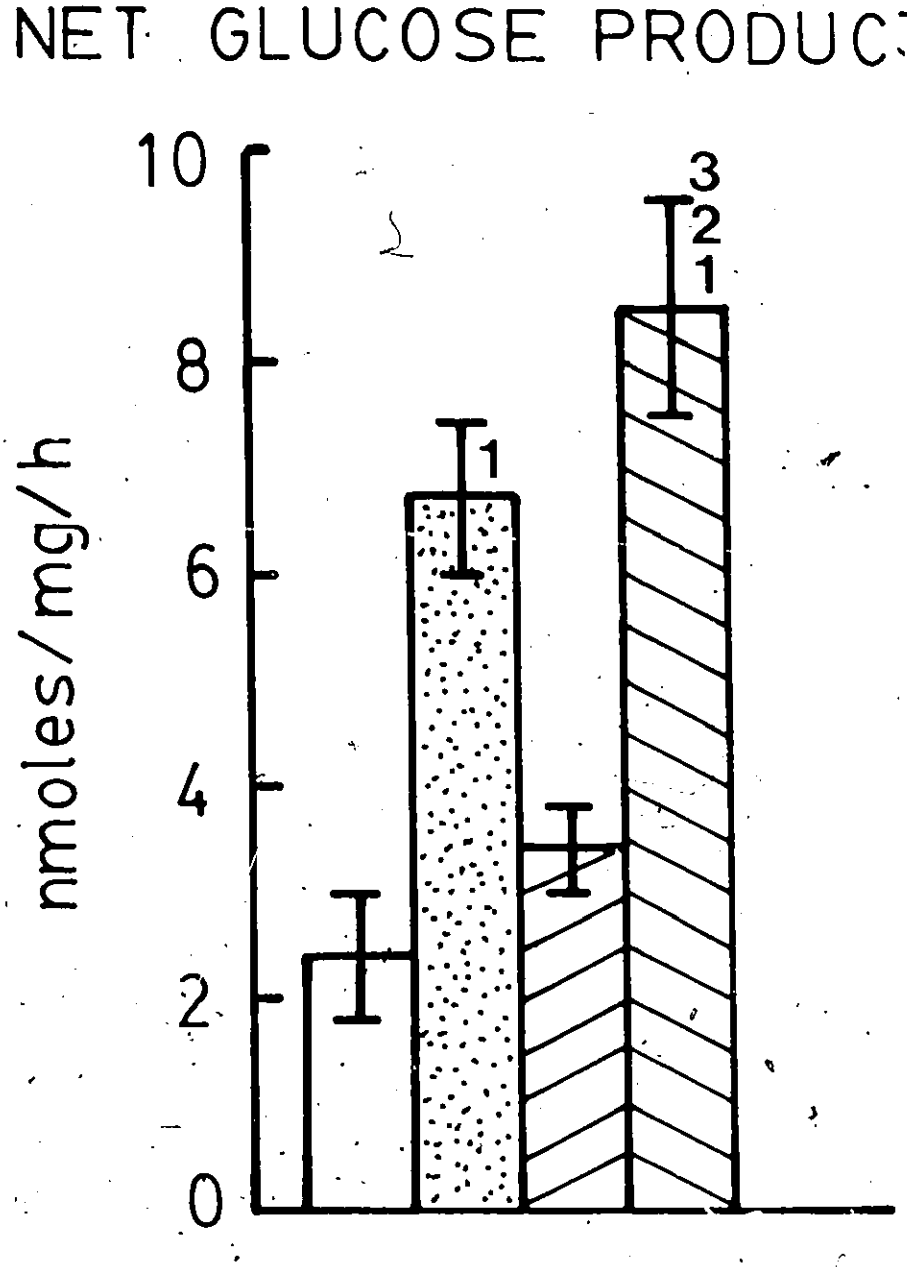

NET GLUCOSE PRODUCTION
nmoles/mg/h
10
8
6
4
2
0
1
3
2
1

Fig. 4-5: The production of glucose (A, n=7), CO_2 (B, n=7), and glycogen (C, n=6) from 5 mM serine as affected by bovine glucagon (10^{-7} M), swordfish insulin (10^{-8}), and glucagon + insulin (10^{-7} and 10^{-8} M, respectively) in sea raven hepatocytes. The experiments were performed in the spring. Protocol and statistics as in Fig. 4-4. Level of significance at $p<0.05$. 1) different from no hormone; 2) different from glucagon; 3) different from insulin.

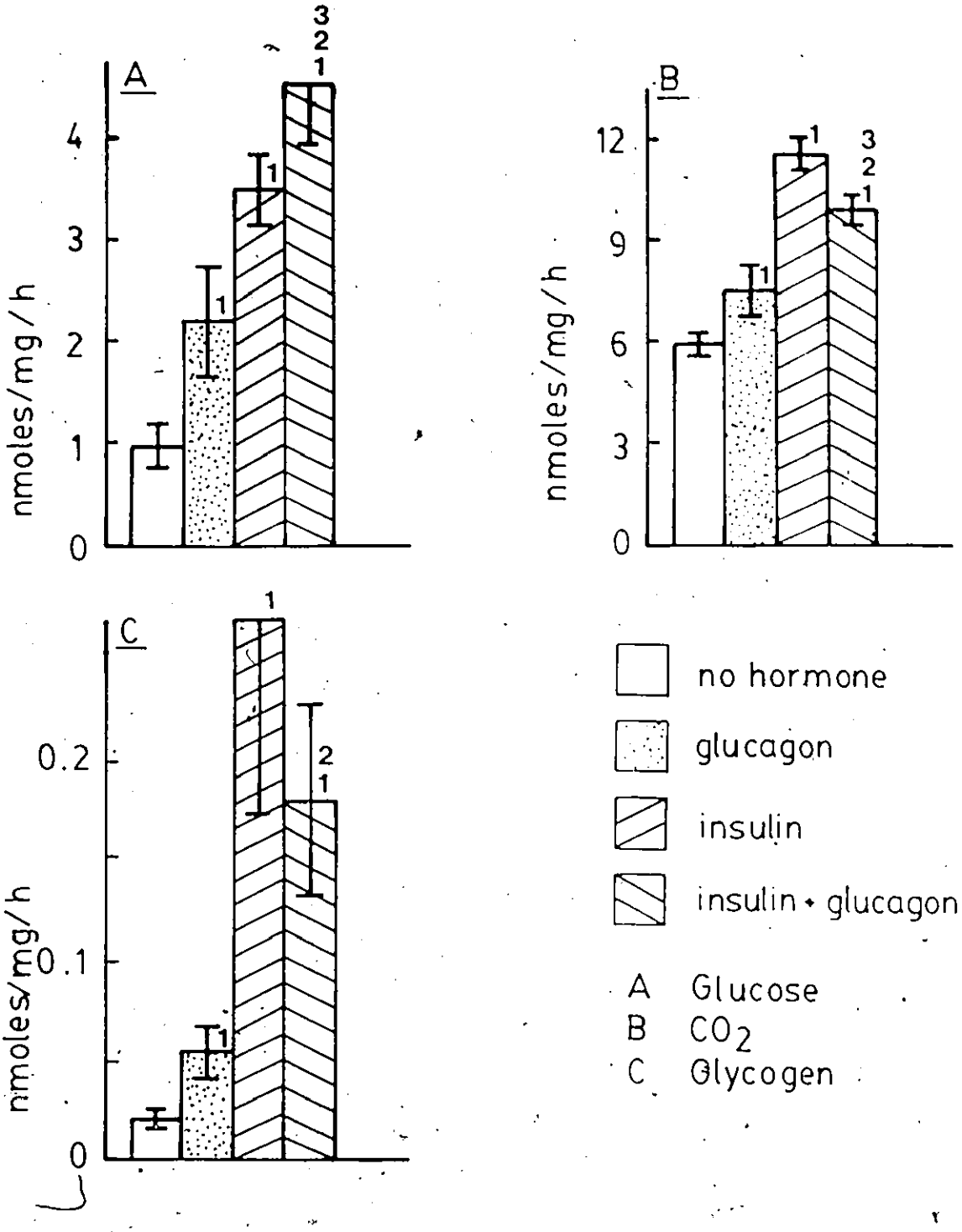

A
B
C
nmoles/mg/h
0
1
2
3
4
6
9
12
0.1
0.2
no hormone
glucagon
insulin
insulin + glucagon
A Glucose
B CO_2
C Glycogen

Table 4-2: Phosphofructokinase (PFK) activity ($umoles.min^{-1}.g^{-1}$ dry wt.) in sea raven hepatocytes with fructose 2,6-bisphosphate (F2,6-P). Hepatocytes were pre-incubated in FRM for 3 h, rinsed, and incubated for a further 2 h. The cells were homogenized and enzyme assays were performed on the highspeed supernatants. F2,6-P was added to the enzyme assay. Activities were estimated at low (0.4 mM) and high (2.0 mM, V_{opt}) F6-P concentrations. The activity ratio is the ratio of these two activities. Significant differences were estimated by paired t-tests at $p<0.05$ (*).

	NO ADDITION	10 uM F2,6-PHOSPHATE
Vopt	0.51 ± 0.10	0.96 ± 0.12*
Activity Ratio	0.11 ± 0.04	0.84 ± 0.05*

Table 4-3: Optimal activities (V_{opt}; umoles.min^{-1}.mg^{-1} dry wt.) and activity ratios of phosphfructokinase (PFK), pyruvate kinase (PK), and phosphenolpyruvate carboxykinase (PEPCK) as affected by porcine and swordfish insulins. Protocol as on Fig. 4-1. Significant differences determined by paired t-tests at $p<0.05$ (*).

HORMONE	ENZYME	Parameter	n	No Hormone	10^{-9} M	10^{-8} M
PORCINE	PFK	V_{opt}	8	0.62 ± 0.08	0.60 ± 0.09	-
		Act. Ratio	8	0.15 ± 0.04	0.20 ± 0.04*	-
	PK	V_{opt}	7	1.44 ± 0.10	1.36 ± 0.13	-
		Act. Ratio	7	0.31 ± 0.02	0.35 ± 0.04	-
	PEPCK	V_{opt}	8	0.13 ± 0.01	0.14 ± 0.02	-
SWORDFISH	PFK	V_{opt}	5	0.84 ± 0.06	0.81 ± 0.06	0.83 ± 0.09
		Act. Ratio	5	0.19 ± 0.06	0.20 ± 0.06	0.17 ± 0.05
	PK	V_{opt}	5	1.28 ± 0.13	1.23 ± 0.11	1.26 ± 0.10
		Act. Ratio	5	0.32 ± 0.02	0.29 ± 0.01	0.30 ± 0.03
	PEPCK	V_{opt}	5	0.19 ± 0.06	0.20 ± 0.03	0.19 ± 0.04

activity ratio, implying a decrease in the K_a for F6-P (Table 4-2). Teleost insulin had no effect on the maximal activity or activity ratio of the three enzymes tested, while mammalian insulin resulted in a 33% increase in the activity ratio of PFK, with no other apparent effects (Table 4-3). High control activities prevented the estimation of FBPase in sea raven hepatocytes.

DISCUSSION

This study demonstrates that the actions of insulin on the sea raven hepatocyte system are different from that previously noted in the mammalian liver. The hormone increased glucose production (Fig. 4-2) and increased the flux of serine to both glucose and glycogen (Fig. 4-3 and 4-5). The only change in glucose utilization was an enhancement of flux to glycogen (Table 4-1). As total glucose utilization accounted for less than 1% of the added substrate this effect is considered to be minor. Serine oxidation was stimulated (Fig. 4-5), in contrast to insulin effects on PDH activities in the rat liver (Weinberg and Utter, 1980), and three key enzymes were unaltered or only slightly altered by insulin (Table 4-3). This latter observation suggests that the mechanism of insulin action in the teleost liver is not the same as that proposed for

the mammalian liver. Also, insulin did not reverse the glucagon-stimulated gluconeogenesis. Rather, it acted additively upon the glucagon effect (Fig. 4-5). As glucose flux changes were minor, insulin appears not to act through this substrate to accomplish its role in these hepatocytes, consistent with the unidirectional glucose-producing capacity of the sea raven hepatocytes (see Chap. 3) and other teleost livers (Cowey and Sargent, 1979).

The increased flux of serine to glucose and glycogen (Fig. 4-3 and 4-5) indicates insulin is augmenting amino acid conversion to carbohydrates in sea raven hepatocytes, while the hormone does not alter glucose utilization (Table 4-1). These results demonstrate two potential functions of insulin in this preparation. Firstly, as the swordfish insulin did not maintain glycogen levels (Fig. 4-1), the prime function of this insulin may be to increase glucose production for export. The sea raven is a sit-and-wait predator that employs its primarily white muscle to dart after prey, and carbohydrates that fuel this activity can only arise from glucose synthesized through the gluconeogenic pathway. The increased incorporation of serine radioactivity into glycogen (Fig. 4-3 and 4-5) could then be attributed to an increased availability of glucose-6-phosphate from serine and the saturation of the glucose-6-phosphatase reaction. Secondly, the maintenance of the hepatocyte glycogen content in the presence of porcine insulin (Fig. 4-1) suggests a second function for

insulin, that of enhancing the glycogen stores. Plisetskaya et al. (1984) reported increased salmon hepatocyte glycogen levels with exposure to salmon and bovine insulin, and similar effects were noted in *Notemigonus chrysoleucas* (de Vlaming and Pardo, 1975) and catfish (Ottolenghi et al., 1985). The present study, using the sea raven hepatocyte system supports the literature and implies that insulin functions in hepatocytes to increase glycogen stores, in addition to increasing glucose production. Further work is required to understand the differential effects of the two insulins on the metabolism of the sea raven hepatocytes, as well as to determine the relative importance of the insulin effect on gluconeogenesis and its glycogen effect. Regardless of the differential actions on glycogen and glucose, it is clear that insulin increased carbohydrate production from amino acids. It should not be overlooked that, and consistent with the observed effects of insulin on sea raven hepatocytes, amino acids are a more potent secretagogue of insulin than is glucose in a number of teleost species (Patent and Foa, 1971; Plisetskaya et al., 1975; Ince and Thorpe, 1977a; Ince, 1979, 1980). Also, Plisetskaya et al. (1984) have reported increased glucose levels in salmon hepatocytes exposed to bovine insulin when incubated in the presence of amino acids, consistent with the insulin effect on gluconeogenesis observed in sea raven hepatocytes.

The action of insulin in the sea raven hepatocyte

system was to increase glucose production, an effect also noted for glucagon in this preparation (see Chap. 3). It appears, however, that the mechanism employed by the two hormones to increase glucose production are different. Glucagon increased glycogenolysis (Fig. 3-1), offset the insulin-induced increase in serine glycogenesis (Fig. 4-5), and stimulated the use of an amino acid as a substrate for glucose production (Fig. 4-3 and 4-5). Thus, the actions of glucagon are upon storage carbohydrate and gluconeogenesis from amino acids (resulting from muscle breakdown?), and appear ideally suited to the regulation of glucose production during periods of food-deprivation. The effects of insulin appear to be basically anabolic, as would be appropriate under feeding conditions.

Glucagon generally depressed the activity of the glycolytic enzymes assayed, and activated PEPCK (Table 3-3). Insulin, on the other hand, affected the activity of only PFK, and that only marginally (Table 4-3). Thus, even though both hormones stimulated gluconeogenesis, these data imply that different mechanisms are used. Insulin increases amino acid transport in mammalian hepatocytes (Freychet et al., 1979), and amino acid uptake in liver slices of the eel (Inui and Ishioka, 1983) and salmon hepatocytes (Plisetskaya et al., 1984). This effect of insulin on membrane transport could prove to be the critical point of insulin action on gluconeogenesis in sea raven hepatocytes.

Porcine insulin had only minimal effects on PFK

(Table 4-3), yet the addition of F2,6-P to the crude enzyme homogenate increased the activity ratio by nearly 8-fold (Table 4-2). Thus F2,6-P may be an allosteric activator of the sea raven enzyme, as in the mammalian liver (Hers et al., 1982). Further studies involving the determination of insulin effects on the sensitivity of the enzyme to F2,6-P, and changes in the intracellular concentration of this metabolite, are neccesary to define the effects of insulin in this system.

The generalized action of insulin on sea raven hepatocytes is an increase in serine glycogenesis and gluconeogenesis, but differential effects of porcine and swordfish insulin, as noted above, were found. Although there is no explanation for these differential effects, it is apparent that the effects of the porcine insulin are more comparable to the carbohydrate effects generally reported in mammals. In addition, these *in vitro* insulin effects are quite different from those reported by Thorpe (1976). He found that a teleost (cod) insulin injected into an intact teleost decreased blood glucose levels, whereas bovine insulin was without effect on this parameter. These differences between the *in vivo* effects reported by Thorpe (1976) and the *in vitro* data presented here, imply that a complex interaction occurs between hormones *in vivo*, and/or that the relationship between liver glucose production and its uptake by peripheral tissues (e.g. skeletal muscle) may be tightly

regulated *in vivo*.

A major effect of insulin in other systems, including both teleosts (Ince and Thorpe, 1976; Ablett et al., 1981; Plisetskaya et al., 1984) and mammals (Poli et al. 1981), is a stimulation of protein synthesis. A small, but significant, increase in serine incorporation into protein was noted in the sea raven hepatocyte system, but only at concentrations above those eliciting the carbohydrate effects (Fig. 4-3). Plisetskaya et al. (1984) found a similar concentration dependant effect in the salmon hepatocyte preparation. These differential effects may indicate the presence of more than one membrane-receptor binding insulin or insulin-like substances in the teleost liver. King and Kahn (1981) have identified dual insulin receptors which modulate the metabolic and growth-promoting effects of insulin in the rat epididymal preparation. In addition, they demonstrated different sensitivities of species-specific insulin for these two effects of insulin. Bonito (a pelagic teleost) insulin showed 50% of the metabolic effect, but 160% of the growth-promoting effect of porcine insulin in their standard preparation. More studies are required to elucidate the role of insulin in protein synthesis in the teleost liver.

Seasonal differences were found in both glucose flux to CO_2 and protein and serine flux to CO_2, glucose, and protein. Oxidation rates of serine and

glucose, and serine gluconeogenic rates during the autumn spawning season were above the summer values (Table 4-1 and Fig. 4-3), while the flux of glucose and serine to protein showed the opposite trend. These data imply a metabolic increase and protein biosynthetic rate decrease during spawning. All flux patterns were depressed in the spring experiments compared to the autumn rates (Fig. 4-5). Spawning during the autumn would place a high demand on metabolism, and thus increase oxidation rates over summer values (Fig. 4-3). The decreased protein synthesis during the reproductive phase indicates energy is directed away from growth to provide energy for reproduction. The diminished serine flux observed in the spring experiments (Fig. 4-5 compared to Fig. 4-3) may be part of an annual cycle, with metabolism still depressed even after water temperatures have started to rise in the spring.

In summary, insulin increased carbohydrate production (glucose and glycogen) in sea raven hepatocytes. Glucose utilization was unaffected by insulin, except for a small increase in flux to glycogen. The gluconeogenic effect of insulin was additive with glucagon, whereas glucagon offset the stimulation of serine glycogenesis and oxidation by insulin. The mechanism of action of insulin does not appear to involve PFK, PK, or PEPCK, but may involve changes in the membrane transport of amino acids. Based on these findings, it is proposed that insulin is important for glucose production, and possibly glycogen

deposition, during periods of feeding, whereas the role of glucagon is to increase glucose production during periods of fasting. Further studies of the mechanism of energy storage in the liver and muscle would better define the role of insulin in these animals. Studies of substrate uptake and protein synthesis, and measurements of other enzyme activities would be useful to identify the point(s) of insulin action in the sea raven hepatocyte scheme.

CHAPTER 5

CONCLUDING DISCUSSION AND SUMMARY

The actions of insulin on the metabolism of teleost fishes are far from clear (e.g. Plisetskaya et al., 1984). Based upon *in vivo* studies of carnivorous species, the role of insulin has been suggested to be on protein and lipid rather than carbohydrate metabolism. King and Kahn (1981) found greater growth-promoting effects of a teleost (bonito) insulin than with porcine and bovine insulin in the rat epididymal cell assay. *In vivo* radioisotope studies have shown that insulin administration increased ^{14}C-glucose flux to liver lipid and muscle lipid and protein, with no change in flux to liver or muscle glycogen (Ince and Thorpe, 1976; Ablett et al., 1981b). As noted in Chapter 1, the *in vivo* study of hormones is complicated by many factors, some of which can be eliminated by an *in vitro* system (Moon et al., 1985). *In vitro* studies using teleosts have demonstrated an increased glycogen content in liver slices and perfused livers after insulin exposure (de Vlaming and Pardo, 1975; Ottolenghi et al., 1985), plus an increased ^{14}C-glucose flux to glycogen (Ottolenghi et al., 1984). This latter effect, however, is not universal (Renaud and Moon, 1980). No studies have specifically determined the role of insulin on the gluconeogenic pathway and, in fact, most studies have used amino acids which are

poor gluconeogenic substrates (Ince and Thorpe, 1976; Ablett et al., 1981b). The present study, using serine as a substrate and radioactive tracers, has found a strong positive effect of insulin on the gluconeogenic pathway. Although this result was not expected based upon the mammalian literature, it is consistent with the enhanced glucose output found in this study (Fig. 4-2) and in salmon heptocytes as reported by Plisetskaya et al. (1984). While increased glucose production is an apparent function of insulin in sea raven hepatocytes, its role on glycogen metabolism remains obscure. Only porcine insulin increased the glycogen content above controls (Fig. 4-1) in contrast to the results of Plisetskaya et al. (1984), where both bovine and salmon insulins increased the glycogen content of the salmon hepatocytes. Even though more studies are neccesary to define the precise mechanism, it is apparent that insulin has a major effect on carbohydrate metabolism in sea raven hepatocytes, albeit in a manner distinct from mammals.

The two previous chapters have shown that both glucagon and insulin increase glucose production in sea raven hepatocytes, but the mechanisms involved are apparently different. Glucagon was shown to stimulate glycogenolysis, increase gluconeogenesis through altering key regulatory enzyme activities, and offset the insulin-stimulation of serine glycogenesis. In contrast, the main action of insulin was to increase gluconeogenesis

with no apparent effect on glycogenolysis. Furthermore, the insulin stimulation of gluconeogenesis was accomplished through mechanisms (e.g. amino acid transport) which apparently did not involve the regulatory enzymes PFK, PK, and PEPCK. Neither hormone affected glucose utilization, and this finding is consistent with the unidirectional character of the carbohydrate pathways of the teleost liver.

These results suggested two proposals for the separation of the actions of glucagon and insulin. Firstly, glucagon increases plasma glucose levels through the enhancement of gluconeogenesis and glycolysis, which is a suitable strategy during periods of fasting. Secondly, insulin increases gluconeogenesis from newly-absorbed amino acids in a feeding fish, for the production of glucose, the substrate neccesary for energy storage in peripheral tissues. Insulin may also function to increase glycogen stores in the hepatocytes. The actions of insulin and glucagon appear to accomplish the same catabolic and anabolic functions, respectively, as they perform in mammals. The mechanisms, however, are well-suited to the biochemical capabilities and requirements of the animal.

It must be kept in mind that the endogenous insulin and glucagon of the sea raven were not used in these studies. For the reasons stated in Chapter 1, however, the effects of the swordfish insulin and bovine glucagon probably reflect the effects of the endogenous hormones,

but caution must be applied to the interpretation of these data until species-specific hormones are available and the quantification of the circulating hormones in fish can be made. The techniques for such studies are being developed, and they promise to provide interesting results. It could be argued that since the carbohydrate effects of the mammalian insulin (e.g. the small change in the activity ratio of PFK (Table 4-3) and the maintenance of glycogen content (Fig. 4-1) resemble the situation in mammals more than does the swordfish insulin, the carbohydrate effects present with the bovine glucagon may be greater than that which would be found with a teleost glucagon. One can postulate, however, that there may have been no evolutionary pressure on glucagon to change its function in the liver. The vertebrate liver has a consistent glucose-producing capacity (e.g. fish (Cowey and Sargent, 1979), amphibians (Brown et al., 1975), and birds (Dickson and Langslow, 1978)), and a recent study demonstrated that glucose is important in regulating glucagon secretion in the A-cells of the European eel (Ince and So, 1984). Insulin, on the other hand, may have evolved more dramatically as the function of the liver changed to included glucose homeostasis ("glucostat", see below).

Teleost insulin did not effect the activity of the enzymes tested (Table 4-3), thus the factor responsible for the increased serine flux to glucose must reside elsewhere. Also, where low control rates of serine flux were found

(the spring studies; Fig. 3-5 and 4-5), glucagon resulted in increased flux to all the endproducts measured, effects not apparent when control rates were higher (Fig. 3-3 and 4-3). Both observations could be explained by effects on amino acid uptake. Insulin and glucagon increase amino acid transport in eel liver slices (Inui and Ishioka, 1983) and mammalian hepatocytes (Freychet et al., 1979). The increase in gluconeogenesis and other rates estimated in the spring study may be related to amino acid uptake as the limiting factor on which the hormones act. Alternatively, it may be due to priming and/or increased sensitivity of the receptors in the spring. Uptake studies, and in particular estimates of seasonal changes in transport rates would help clarify this issue.

Serine was the chosen amino acid substrate for the studies in this thesis. In all carnivorous vertebrates examined to date, serine is metabolized to glucose in the liver through a pathway that bypasses PEPCK (Rowsell et al., 1973, 1974; Walton and Cowey, 1979b). This may be the situation in the sea raven. In the rat, serine-pyruvate transaminase (SPT) is the first committed step in serine metabolism and this enzyme is stimulated by glucagon and by alloxan administration (Rowsell et al., 1973; Snell and Walker, 1974). No studies of insulin effects on this pathway in teleosts have been reported to date, to my knowledge, but certainly it should be investigated.

As discussed above, glucagon increases glucose

production as in mammals, while insulin effects are different than in mammals. These conclusions are consistent with the apparent capabilities of the teleost liver (Cowey and Sargent, 1979) including that of the sea raven (see Chap. 3), and they raise some interesting suggestions with respect to evolution and environmental physiology.

The rat liver has a glucostatic function, with insulin increasing glucose utilization in the tissue (Reiser, 1967), and glucose is the main secretagogue of the hormone. In contrast, the teleost liver apparently has no glucostatic function (Cowey and Sargent, 1979), insulin does not increase glucose utilization (Table 4-1), and in a variety of teleosts, amino acids are the prime secretagogue of insulin (Patent and Foa, 1971; Plisetskaya et al., 1975; Ince and Thorpe, 1977a). There is an apparent correlation here with both diet and metabolic activity. Insulin effects may be related to the protein diet of the sea raven (see Chap. 4) but, alternatively, it may be related to the low metabolic activity of the sea raven. Severe depression of blood glucose in the rat will cause convulsions and death; a similar response occurs in relatively active teleosts but not inactive species (Gray, 1928; Gray and Hall, 1930; Leibson, 1968). Active organisms have a greater demand for glucose and rely on the blood supply to provide this metabolite (Leibson, 1968). These species maintain blood glucose concentrations at a higher level than inactive ones (Umminger, 1977) and more tightly regulate its content.

(Leibson, 1968). The liver in these species may have a significant glucostatic role. While a glucostatic function is not apparent in the more active teleosts like the salmonids (Cowey and Sargent, 1979), extremely active fish, such as the continuously swimming tunas, may have evolved this capability. Studies of liver carbohydrate metabolism and insulin effects in these organisms could prove to be valuable and provide some insight into the development of glucose regulatory systems that have enabled the development of, or are a result of, the high metabolic rates found in homeothermic organisms.

Summary. Previous to the present work, little was known about the mechanisms involved in glucagon-enhanced glucose production, the mechanism of action of insulin, and the degree of antagonism that exists between the two hormones in a teleost liver. Chapter 1 stated the reasons for using an *in vitro* preparation rather than the intact animal, and a successful hepatocyte preparation was obtained from the sea raven. In this manner, the confounding factors in a more complex system were removed.

In response to the questions asked in the Chapter 1 the following answers can now be provided:

(1) are the effects of glucagon in the sea raven hepatocytes similar to the effects in other teleosts? The overall effects of glucagon in sea raven hepatocytes resemble those found in most teleost species.

(2) is glycogen a precursor in glucagon-stimulated glucose production?

Glycogen, as well as gluconeogenic substrates, has a role in the glucagon-enhanced glucose production in sea raven hepatocytes.

(3) what are the effects of insulin on carbohydrate metabolism in sea raven hepatocytes?

Insulin effects anabolic changes in sea raven hepatocytes by increasing the flux of serine to glucose and glycogen. The enhanced gluconeogenic flux may be part of a mechanism to increase glucose production for storage in glucose-utilizing tissues.

(4) what degree of antagonism exists between insulin and glucagon?

Glucagon and insulin function antagonistically with respect to oxidation and glycogen metabolism, but not within the gluconeogenic/glycolytic pathways. In fact the effects on these pathways are additive. The mechanistic effects on the pathway are different, with glucagon altering PFK, PK, and PEPCK activities. Insulin did not affect any of these enzymes.

(5) are there differential effects of a teleost and mammalian insulin in sea raven hepatocytes?

Whereas porcine insulin maintained glycogen levels, did not alter glucose values, and increased the activity ratio of PFK, the swordfish insulin did not protect glycogen from degradation, increased glucose production, and did not

alter PFK activities. Both insulin types resulted in similar effects on serine flux.

Various experiments required to further elucidate these studies have been suggested throughout the thesis. Certainly the findings of this thesis indicate that future work will prove to be exciting.

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