THE HORMONAL CONTROL OF HEPATIC METABOLISM IN THE RAT.

A thesis submitted for the degree of:

Doctor of Philosophy,

in the

University of London.

by:

Christopher John Kirk,

Department of Biochemistry,

Imperial College of Science and Technology,

London, S.W.7 2AZ.

October 1977.

Abstract

The hormonal control of hepatic metabolism in the rat was studied in vivo and in vitro with particular reference to the role of adrenal glucocorticoids and 8-arginine vasopressin, which are implicated in stress responses. Rat livers were perfused in situ, and rates of de novo fatty acid synthesis were measured by the incorporation of ^{3}H from $^{3}H_2O$ and ^{14}C from ^{14}C labelled substrates into tissue lipids. Gluconeogenesis and glycogenolysis were followed by the appearance of glucose in the perfusion medium and the depletion of hepatic glycogen levels.

The de novo synthesis of fatty acids in the perfused liver, their incorporation into hepatic glycerides and their release into the perfusion medium were all diminished following adrenalectomy in the rat. Adrenal ablation also led to a reduction in the proportion of monoenoic fatty acids amongst those synthesized de novo in perfusions. The diminished incorporation of newly synthesized fatty acids into hepatic glycerides following adrenalectomy did not appear to be due to any decreased capacity for esterification since the perfused livers of adrenalectomized rats incorporated exogenous circulating fatty acids into hepatic glycerides at the normal rate.

Fatty acid synthesis, desaturation and release from the perfused livers of adrenalectomized rats were restored to normal rates following 5 h. treatment with cortisol in vivo, but not in vitro. These results are discussed in terms of a possible role for insulin in the control of hepatic lipogenesis in the intact animal by glucocorticoids.

The peptide hormone 8-arginine vasopressin was shown to promote gluconeogenesis and glycogenolysis in the perfused livers of fasted and fed rats respectively. This latter effect was dependent upon the presence of adrenal hormones, since the glycogenolytic potency of the hormone was much reduced in the perfused livers of adrenalectomized rats. Vasopressin-stimulated glycogenolysis in the perfused liver was inhibited by the presence of 5 mU/ml insulin in the perfusion medium.

The effect of vasopressin upon the concentrations of various tissue intermediates was studied in the perfused livers of fed and fasted rats, and the results of this study indicate a facilitation of the conversion of pyruvate to phosphoenol pyruvate and of glucose-6-phosphate to glucose. There was also a very marked drop in α-ketoglutarate concentration in the hormone-treated livers.

Vasopressin failed to influence the total rate of fatty acid synthesis in the perfused rat liver, or the contribution of lactate carbon to that rate. The complete oxidation of circulating lactate to CO_2 was also unaffected by the hormone. Vasopressin failed to influence ketogenesis from circulating oleate in the perfused liver.

The measurement of the hepatic concentrations of cyclic nucleotide monophosphates and their release into perfusion medium following hormone treatment failed to indicate a role for these nucleotides in the hepatic actions of vasopressin. In similar experiments, glucagon and adrenalin produced the expected rise in the accumulation and release of hepatic cyclic AMP.

The influence of extracellular Ca^{++} concentration and temperature upon the release of glucose by the perfused livers of fed

rats was studied in perfusions with and without vasopressin. The results of these experiments indicated that both hormone and heat stimulated glucose release from the perfused livers of fed rats are influenced by the extracellular concentration of Ca^{++}. The possible mechanism of action of vasopressin is discussed in the light of these findings.

Acknowledgements

I should like to thank my supervisor Dr. D. A. Hems for his encouragement and helpful discussions during the past years. My gratitude is also due to all my colleagues at Imperial College who helped to make my time there so enjoyable. Particular thanks should go to Mr. T. R. Verrinder, whose technical expertise played a major role in my initiation into the mysteries of lipid metabolism. The staff of the Animal Unit have been particularly long-suffering during my stay at the College, and I am grateful for all their help.

Finally, I should like to thank Mrs. A. Kirk and Mrs. O. Bridges who typed the manuscript, my parents for their constant support, and my wife Debby who has helped and encouraged me as only she is able.

CONTENTS

Abbreviations

Units and Physical Constants.

The units and physical constants used in this thesis (and the appropriate abbreviations) are as recomended in Biochem. J. (1975) 145, 1.

Chemicals.

Unless listed below, all abbreviations used in this thesis are as recomended in Biochem. J. (1975) 145, 1.

ACTH	Adrenocorticotrophic hormone.
Butyl PBD	5-(4-biphenylyl)-2(4-t-butylphenyl)-1-oxa-3,4-diazole.
FA	Fatty acid.
FAME	Fatty acid methyl ester.
PEPCK	Phosphoenolpyruvate carboxykinase.
PL	Phospholipid.
PLFA	Phospholipid fatty acid.
TCA	Trichloracetic acid.
TG	Triacylglycerol.
TGFA	Triacylglycerol fatty acid.
U	Units.
VLDL	Very low density lipoprotein.

List of Tables

List of figures

18 The effect of vasopressin upon gluconeogenesis in the perfused liver of a 48 h. fasted rat.

19 The effect of vasopressin upon glucose production by the perfused livers of 48 h. fasted rats.

20 The interaction between vasopressin and insulin in the control of glucose release by the perfused liver.

21 The effect of vasopressin upon the concentrations of intermediates in the perfused livers of 48 h. fasted rats.

22 The effect of vasopressin upon the concentrations of intermediates in the perfused livers of fed rats.

23 The influence of vasopressin upon the hepatic release of ketone bodies into perfusion medium.

24 The effect of hormones upon hepatic cyclic AMP in vivo.

25 The effect of hormones upon effluent perfusate cyclic AMP from the perfused liver.

26 The effect of hormones upon hepatic glucose output.

27 The time course of glycogen phosphorylase activation by glycogenolytic hormones in vivo.

28 The effects of Ca^{++} concentration and temperature upon basal and vasopressin stimulated glucose release by the perfused rat liver.

1 Introduction.

1.1 Some General Observations Concerning the Hormonal Control of Intermediary Metabolism.

1.1.1 Metabolic Abnormalities in People with Diseases of the Adrenal Gland.

1.1.2 The Endocrine and Metabolic Response to Stress.

1.2 The Regulation of Lipid Metabolism by Adrenal Corticosteroids.

1.2.1 The Role of Peripheral Fat Mobilisation in the Adrenal Control of Lipid Metabolism.

1.2.2 The Effects of Adrenal Corticosteroids upon Hepatic Fatty Acid Synthesis.

1.2.3 The Effects of Adrenal Corticosteroids upon the Release of Hepatic Lipids.

1.2.4 The Effects of Adrenal Corticosteroids upon Hepatic Cholesterol Synthesis.

1.3 The Role of Adrenal Corticosteroids in the Regulation of Hepatic Carbohydrate Metabolism.

1.3.1 The Involvement of Extra-Hepatic Tissues in the Adrenal Control of Carbohydrate Metabolism.

1.3.2 The Effects of Adrenal Corticosteroids upon Basal Hepatic Gluconeogenesis.

1.3.3 The 'Permissive' Effect of Adrenal Corticosteroids upon the Hormonal Control of Hepatic Gluconeogenesis.

1.3.4 The Effects of Adrenal Corticosteroids upon Hepatic Glycogen Metabolism.

1.4 The Influence of Vasopressin upon Intermediary Metabolism.

1.5 The Mechanism of Short Term Hormone Actions in the Liver.

1.6 Scope and Aims of the Present Study.

1.6.1 The Perfused Rat Liver as a Model for the Study of the Hormonal Control of Hepatic Metabolism.

1.6.2 The Specific Aims of the Experiments Described in the Present Study.

1 Introduction.

1.1 Some General Observations Concerning the Hormonal Control of Intermediary Metabolism.

1.1.1 Metabolic Abnormalities in People with Diseases of the Adrenal Gland.

The importance of the adrenal gland in the regulation of mammalian intermediary metabolism was first indicated by Addison's classical description of adrenal insufficiency in 1855. However, it was not until 53 years later that Bierry and Malloizel (1908) first described hypoglycaemia in adrenalectomized dogs. In the following years a relatively complete picture of the metabolic abnormalities associated with Addison's disease, and their similarities to the effects of adrenalectomy, was assembled. Thus it is now well known that patients with Addison's disease exhibit hypoglycaemia and an abnormal response to the glucose tolerance test, (frequently involving a hypoglycaemic overshoot). This overshoot is symptomatic of the extreme sensitivity to insulin which is associated with the disease. Patients suffering from adrenal insufficiency also exhibit a loss of weight and decreased body fat reserves.

The disease now known as Cushing's syndrome was first described by Harvey Cushing in 1932. The visible features of the disease include obesity of the trunk, face (moon face) and buttocks but not of the limbs. The metabolic abnormalities associated with the disease are well characterised; thus Thorn et. al. (1957) have described fasting hyperglycaemia, abnormal glucose tolerance tests (in which a return to normal glucose levels within 60 - 90 minutes was not observed) and glycosuria in patients with Cushing's syndrome. All of these features are characteristic of classical diabetes mellitus However in contrast to diabetics,

patients with Cushing's syndrome exhibit depletion of body protein out of proportion to the other defects in intermediary metabolism,also relative insulin resistance and no tendency to ketoacidosis.

Cushing's syndrome has been shown to result from excessive production of cortisol, (usually associated with adrenal hyperplasia or adrenal tumours), but aldosterone production remains normal. Thus Cope and Pearson (1965) have reported that cortisol production in people suffering from Cushing's syndrome can be as high as 316 mg./day while the mean normal production is 16.2 mg./day. Migeon et. al. (1963) have shown that obese individuals who are not suffering from Cushing's syndrome may also exhibit moderately enhanced cortisol production.

These clinical observations thus emphasize the profound effects upon intermediary metabolism which may result from alterations in adrenal cortical secretions. There has been considerable work throughout the years involving the use of experimental animals in an attempt to characterise these effects of adrenal corticosteroids.

1.1.2 The Endocrine and Metabolic Response to Stress.

It has long been clear that stressful situations are capable of exerting powerful influences upon metabolic events. In humans for example, surgical operation or physical injury initiates a series of metabolic changes in which protein is catabolised, fat is degraded and oxidised, glucose is mobilised and water and sodium are retained (Browne et. al. 1944; Cuthbertson, 1930). Many of these metabolic changes are attributable to enhanced endocrine activity.

The endocrine response to trauma principally involves the pituitary and adrenal glands. Thus Cooper and Nelson (1962) have shown that stress is associated with a sudden increase in the concentration of plasma adrenocorticotrophic hormone (ACTH) in pre-operative patients. Increased ACTH levels are associated with enhanced secretion from the adrenal cortex, the importance of which may be judged from the fact that adrenalectomized animals are unusually vulnerable to stress of all kinds. In general, the importance of this adrenocortical response to stress may rest with the ability of corticosteroids to permit other hormones to exert their metabolic effects.

Adrenal medullary activity is also enhanced during stress (Cellander, 1954). Carey et. al. (1973) have reported that the plasma concentration of adrenalin rises during haemorrhagic shock, and it is possible that this circulating adrenalin may be responsible for the increased plasma concentrations of glucose, lactate and amino acids which generally accompany such trauma (Sayers et.al.1945;Beatty,1945).

The posterior pituitary gland also has a role in adaptation to stress. Haemorrhagic shock has been shown to induce the release of vasopressin into the circulation (Ginsburg and Heller,1953), which would exert a vital pressor action, and could contribute to hepatic glucose mobilisation (Hems and Whitton,1973). Other stressful stimuli have also been reported to cause vasopressin release. Thus Forsling (1974) has shown that severe hypoxia leads to vasopressin release in dogs. In people, apprehension and fear have been shown to elevate blood levels of vasopressin (Moran et.al.1974), and increased concentrations of the hormone were found in the urine of post-operative patients (Chine et. al. 1953).

In the present study, the metabolic effects of two hormones involved in stress responses, viz. glucocorticoids and vasopressin have been studied in the perfused rat liver.

1.2 The Regulation of Lipid Metabolism by Adrenal Corticosteroids.

1.2.1. The Role of Peripheral Fat Mobilisation in the Adrenal Control of Lipid Metabolism.

Experimental manipulations which produce fatty livers and increase ketogenesis in normal animals have frequently been shown to be less effective in adrenalectomized rats. Thus fatty infiltration of the liver and increased ketogenesis induced by anterior pituitary extract, pancreatectomy, starvation, partial hepatectomy or by exposure to cold can be prevented by adrenalectomy. (Fry 1937; MacKay and Carne 1938; Leblond et. al. 1939). This observation prompted much early work concerning the role of adipose tissue in the overall changes in lipid metabolism which accompany adrenal ablation.

Barnes et. al. (1941) reported that adrenalectomy did not impair the intestinal absorption of dietary fat or its' incorporation into intestinal phospholipids. Schiffer and Wertheimer (1947) reported that the extensive loss of body fat in adrenalectomized rats was corrected if the electrolyte balance of the animals was restored to normal, as was the ability to deposit dietary fat at the depots. Stoerck and Porter (1950) measured changes in the weight of the rat epididymal fat body as an index of fat deposition and depletion in vivo. These workers found that adrenalectomy alone had no effect upon fat body weight. However, diet restricted adrenalectomized rats (in electrolyte balance) lost fat twice as rapidly as intact animals maintained on the same food intake and four times more rapidly than adrenalectomized animals treated with cortisone. Tracht et. al. (1956) measured the extractable fat content of the epididymal fat body and reported that fatty

acid released from this organ in response to fasting and phlorhizin treatment was unaltered following adrenalectomy. In a similar study, Levy and Ramey (1959) found that the fasting adrenalectomized rat mobilised fat from epididymal adipose tissue in response to fasting more rapidly than normal animals. Cortisol treatment elicited a marked inhibition of fat mobilisation in both adrenalectomized and normal animals.

These data indicate that the resistance to hepatic fatty infiltration exhibited by the adrenalectomized rat is not due to an inability to mobilise peripheral fat. Rather, adrenalectomy appears to exert a direct effect upon the rate of liver fat accumulation.

However, in contrast to the results of Levy and Ramey (1959), other workers have reported decreased release of free fatty acid by epididymal and mesenteric adipose tissue from fasted adrenalectomized rats in vitro. (Schotz et. al., 1959; Rechef and Shapiro, 1960). These latter workers noted that pretreatment of the starved donor rats with cortisone increased the release of free fatty acid by the mesenteric adipose tissue of adrenalectomized rats, but had no effect upon release from the adipose tissue of intact rats.

Jeanrenaud and Renold (1960) reported that steroids in vitro increased the net release of free fatty acid from isolated rat epididymal adipose tissue incubated with pyruvate and glucose. In a recent study Mukherjee and Mukherjee (1973) showed that basal lipolysis in rat epididymal fat pads in vitro was significantly reduced following adrenalectomy. These workers reported that cortisol (50 $\mu g.\ 100g^{-1}$) administered 30 minutes prior to sacrifice

stimulated lipolysis and decreased the rate of re-esterification in adipose tissue from both normal and adrenalectomized rats.

The role of the adrenal gland in hormonally stimulated lipolysis has been the subject of considerable investigation, and definitive conclusions are still not at hand. Shafrir et. al. (1960) have reported that adrenalectomy inhibits adrenalin induced lipolysis in rat adipose tissue both in vivo and in vitro. Shafrir and Kerpel (1964) demonstrated that the diminished adrenalin-induced lipolysis in isolated rat epididymal adipose tissue from adrenalectomized rats was restored to normal by cortisol treatment in vivo. These workers found that this "permissive" effect of cortisol was accompanied by reduced glucose uptake and glycogen levels in the adipose tissue. They suggested that the enhanced lipolysis in the cortisol treated animals resulted from reduced esterification of fatty acids following these changes in carbohydrate metabolism.

Corbin and Park (1969) reported that adrenalectomy reduced adrenalin-stimulated lipolysis in isolated epididymal fat pads, but did not alter basal lipolysis or cyclic AMP accumulation. In isolated fat cells the lipolytic response to physiological levels of adrenalin or cyclic AMP was reduced by adrenalectomy and restored after two hours exposure to dexamethasone in vitro. The maximal rates of lipolysis induced by high concentrations of the hormone or cyclic AMP remained unaltered by adrenalectomy. These workers suggested that glucocorticoids modulate the effects of adrenalin on lipolysis by altering the sensitivity of a hormone sensitive lipase to activation by cyclic AMP.

Very recently, Lamberts et. al. (1975) have produced evidence

suggesting that the induction of a cyclic AMP dependent protein kinase by glucocorticoids is responsible for their ability to permit adrenalin-stimulated lipolysis.

Adrenalectomy has also been reported to inhibit both nicotine-(Bizzi et. al., 1972) and ACTH-(Braun and Hechter,1970) induced lipolysis in adipose tissue. These last workers found that the ACTH stimulation of adenylate cyclase in plasma membrane sacs of isolated adipocytes is abolished by adrenalectomy. Administration of glucocorticoids restored the sensitivity to ACTH in a way that was blocked by both actinomycin D and cycloheximide, suggesting that the hormones were increasing the synthesis of a protein component of the hormone-receptor complex.

While the precise role of the adrenal gland in the regulation of peripheral fat mobilisation remains unsettled there is even more disagreement concerning the effects of adrenalectomy or steroid treatment upon fatty acid synthesis in adipose tissue. Welt and Wilhelmi (1950) studied the incorporation of ^{2}H from $^{2}H_2O$ into the liver and carcass fat of adrenalectomized rats, and found that the amount incorporated in both regions was greater than that in normal rats.

Munck and Koritz (1960) reported that the administration of cortisol to fasted adrenalectomized rats slightly decreased the recovery of ^{14}C from orally administered (^{14}C)-glucose in the total lipid of epididymal fat pads removed 35 hours later.

Munck (1961 a, b) observed that a single injection of cortisol (0.5 mg) in adrenalectomized rats reduced the glucose uptake of epididymal fat pads removed for incubation 30 minutes later. Furthermore, both cortisol and corticosterone produced a

substantial decrease in glucose uptake by epididymal adipose tissue from adrenalectomized rats when added to the incubation medium at concentrations greater than 10^{-5}M. With longer incubation periods, lower concentrations of these hormones ($9x10^{-7}$M) reduced the rate of glucose uptake by more than 50% during the period from 2.5 to 5 hours. This effect of glucocorticoids has also been confirmed more recently (Livingston and Lockwood, 1975).

Jeanrenaud and Renold (1960) studied the metabolism of 2-(^{14}C)-pyruvate and U-(^{14}C)-glucose by epididymal adipose tissue from normal and adrenalectomized rats. They found that $^{14}CO_2$ production and the incorporation of ^{14}C into fatty acids were similar in adipose tissue from both normal and adrenalectomized rats providing that comparable nutritional status had been achieved. These workers also showed that cortisol or corticosterone similarly failed to influence $^{14}CO_2$ production or lipogenesis from glucose or pyruvate when added in vitro (30 μg. ml^{-1}). These hormones did, however, significantly increase the net release of free fatty acids from adipose tissue.

Leboeuf et. al. (1962) repeated the work of Jeanrenaud and Renold (1960) with a lower concentration of glucose in the medium, and older rats. These workers reported that the addition of cortisol (30 μg. ml^{-1}) in vitro resulted in a 10-30% decrease in the oxidation of (^{14}C)-glucose to $^{14}CO_2$, and in the incorporation of ^{14}C into glyceride-glycerol,fatty acids and glycogen. In a similar study, Fain et. al. (1963) also observed a depression of glucose uptake, $^{14}CO_2$ production and the incorporation of ^{14}C into lipid and fatty acids of parametrial and mesenteric adipose tissue following dexamethasone treatment (10^{-7}-10^{-8}M) for 2 hours in

vitro.

In view of the contradictory nature of much of the evidence discussed in this section, it is difficult to make any general conclusions concerning the role of the adrenal gland in adipose tissue lipid metabolism. Thus, while the adrenal gland may influence hormonally stimulated lipolysis in adipose tissue, basal lipolysis, at least in the fed state, seems to be relatively unaffected by changes in cortical activity. The general conclusion from the studies concerning fatty acid synthesis in adipose tissue is that the adrenal glucocorticoids tend to marginally inhibit fatty acid synthesis, while adrenalectomy has little effect upon the process. Thus, no strong case can be made for fatty acid synthesis and mobilisation from adipose tissue playing a major role either in the resistance of adrenalectomized animals to hepatic fatty infiltration or to the obesity which develops in Cushing's syndrome. An alternative explanation for these phenomena would be that glucocorticoids stimulate lipogenesis and triglyceride export in the liver. In the present study, experiments have been performed to test this possibility.

1.2.2 The Effects of Adrenal Corticosteroids upon Hepatic Fatty Acid Synthesis.

Measurements of hepatic fatty acid synthesis in the intact adrenalectomised rat have yielded conflicting results. Thus, Welt and Wilhelmi (1950) reported that hepatic fatty acid synthesis, measured with deuterated water, was elevated in the adrenalectomized

rat; while Fain and Wilhelmi (1962) found no change in the rate of fatty acid synthesis from tritiated water following adrenal ablation. More recently, Diamant and Shafrir (1975) reported that the incorporation of (^{14}C)-acetate into hepatic fatty acids in normal rats was increased after 2-5 days treatment with triamcinolone.

Perry and Bowen (1955, 1956) have shown that the incorporation of ^{14}C labelled acetate into fatty acids is diminished in liver slices prepared from adrenalectomized rats. However, large doses of cortisone (5-20 mg. day $^{-1}$ for 3 days) have been reported to inhibit fatty acid synthesis from (^{14}C)-acetate in liver slices (Brady et. al. 1951).

In other tissues, adrenalectomy has been reported to inhibit lipogenesis. Thus, Rhoades et. al.(1974) have demonstrated that fatty acid synthesis from glucose in the rat lung is depressed by 30% following adrenalectomy, and Wheldrake (1972) found that phosphatidyl choline synthesis in the kidney was inhibited in adrenalectomized animals. The rate of phospholipid synthesis was somewhat restored by treatment with desoxycorticosterone.

1.2.3 The Effects of Adrenal Corticosteroids upon the Release of Hepatic Lipids.

Much of the early work on the adrenal control of lipid metabolism involved the measurement of blood lipid levels in response to steroid hormones or adrenalectomy. Thus, Kobernick and

More (1950) showed that in rabbits cortisone treatment over 22 days resulted in increased levels of plasma cholesterol, phospholipids and especially triglycerides. Di Luzio et. al. (1954) demonstrated that plasma phospholipids and cholesterol decreased by 50% within 3-4 weeks in adrenalectomized dogs maintained on deoxycorticosterone acetate.

However, more recently, studies concerning the short-term effects of steroids upon the release of hepatic lipids have also been performed. Klausner and Heimberg (1967) reported that the net release of triglyceride by the perfused rat liver was reduced following adrenalectomy, while the uptake of free fatty acids by these livers remained unchanged and the output of ketone bodies was marginally stimulated. Treatment of the adrenalectomized rat with cortisone in vivo restored the triglyceride output of the subsequently perfused liver to normal. The addition of large amounts of cortisol to the perfusion medium stimulated net triglyceride release in the perfused livers of both normal and adrenalectomized rats. This effect of cortisol was only observed within a limited dose range.

A recent paper from Reaven et. al. (1974) indicated that in methylprednisolone-induced hyperlipoproteinemia in rats and mice there is an increase in the number and size of Golgi associated very low density lipoprotein particles in hepatocytes. These workers also report that corticosteroid treatment over 8 days results in increased plasma triglyceride and cholesterol levels in normal animals, and also an increase in the rate of hepatic triglyceride accumulation following inhibition of very low density lipoprotein removal by Triton. These results may be

interpreted to suggest that corticosteroids induce hyperlipoproteinemia through increased hepatic production of very low density lipoproteins.

Kyner (1972) has recently reported that in the short term (2-8 hours), steroids reduce blood triglyceride levels in the offspring of normal and diabetic people with no concomitant change in the levels of circulating cholesterol, free fatty acid, insulin or growth hormone. Kyner postulated that this short-term action of steroids represents a direct effect upon the liver. Thus it seems that hepatic triglyceride release in people is not normally stimulated by steroids in the short term as it can be in the perfused liver.

In the present study, the effect of adrenal ablation and steroid replacement upon the release of newly formed fatty acids (in triglycerides) from the perfused liver has been studied in parallel with the measurement of hepatic fatty acid synthesis de novo.

1.2.4 The Effects of Adrenal Corticosteroids upon Hepatic Cholesterol Synthesis.

As indicated in the last section, there has long been considerable interest in the effects of corticosteroids upon the level of circulating cholesterol. This interest partly arose from the possible implication of this circulating lipid in the development of various conditions such as obesity, atherosclerosis and coronary heart disease.

Early work on hepatic cholesterogenesis in liver slices from adrenalectomised rats produced conflicting results. Thus, Perry and Bowen (1956) reported that cholesterol synthesis from (^{14}C)-acetate was reduced by 40% in liver slices following adrenalectomy; while Willmer and Foster (1960) found a 50% rise in hepatic cholesterogenesis in similar experiments in which steroid treatment for seven days had no significant effect. These latter workers reported that the process remained enhanced following steroid treatment of adrenalectomized rats prior to the preparation of liver slices. Only in the case of deoxycorticosterone acetate treatment was the rate of cholesterol synthesis increased above the level observed following adrenalectomy alone. In the normal rat, treatment with this hormone increased the rate of cholesterogenesis in subsequently prepared liver slices.

There is other evidence which suggests that adrenal corticosteroids might play a role in hepatic cholesterogenesis. Thus, in the rat, Scheving and Pauly (1966) have shown that serum corticosteroids exhibit a diurnal rhythm, the greatest concentrations being reached 6 hours before the highest rates of hepatic cholesterol synthesis are found.

Hickman et. al. (1972) have reported that the circadian rhythm of cholesterol synthesis from acetate in liver slices is abolished following adrenalectomy, and that the conversion of acetate to cholesterol occurs at an elevated rate similar to that exhibited by slices from intact rats killed at the middle of the dark phase.

Huber et. al. (1972) have reported that the circadian rhythm of the microsomal enzyme β-hydroxy-β methylglutaryl-CoA reductase is unaffected by adrenalectomy. In contrast to this report,

Edwards (1973) demonstrated that the circadian rhythm of activity normally exhibited by this enzyme was abolished following adrenalectomy, enzyme activity remaining at a low level. In agreement with Huber et. al. (1972), Edwards found that the cyclical feeding pattern and total daily food intake was unaffected by adrenal ablation. Hormone replacement with large single doses of steroids had little effect upon reductase activity.

In a recent study, Nervi and Dietschy (1974) measured cholesterogenesis from(^{14}C)-octanoate in liver slices prepared from rats kept under carefully controlled dietary intake and cyclic lighting conditions. These workers investigated the influence of lighting, cholestyramine feeding, fasting, lipoprotein injection and stress upon cholesterol synthesis in liver slices from normal and adrenalectomized rats. In all cases, both groups of animals exhibited similar rates of cholesterol synthesis, thus suggesting that the adrenal gland is not involved in the regulation of cholesterol synthesis, at least in liver slices.

There is thus conflicting evidence regarding the role of the adrenal gland in the control of hepatic cholesterol synthesis, both in regard to the qualitative nature of the changes involved and in that measurements of synthesis are not compatible with assays of the enzyme which is widely agreed to be rate limiting. This inconsistency obtains, despite elaborate efforts to control variables such as feeding and lighting conditions.

In the present study it was of interest to study the influence of the adrenal gland upon hepatic cholesterogenesis de novo not merely to contribute to the understanding of the hormonal control of lipid biosynthesis, but also because

cholesterol is one of the components of the very low density lipoprotein (VLDL) implicated in the export of hepatic triglyceride.

1.3. The Role of Adrenal Corticosteroids in the Regulation of Hepatic Carbohydrate Metabolism.

The ability of adrenal corticosteroids to maintain body carbohydrate stores by stimulating gluconeogenesis from protein was first systematically demonstrated by the classic work of Long et. al. (1940). These workers showed that the administration of adrenal cortical extract (or crystalline glucocorticoid preparations)to fasted normal or adrenalectomized rats elevated hepatic glycogen concentrations and caused an associated increase in nitrogen excretion. This work extended the findings of Evans (1936) who demonstrated that fasted adrenalectomized animals excreted 25% less nitrogen than normal controls. About the same time, Lewis et. al. (1940) showed that cortisone greatly increased the renal excretion of glucose and nitrogen in adrenalectomized phlorizin treated rats. These workers also demonstrated that when a lactate load was administered to phlorizin treated normal adrenalectomized and cortisone injected rats, urinary glucose excretion accounted for 71% of the administered lactate in the normal group; 80% in the hormone treated group, but only 26% in the untreated adrenalectomized phlorizin treated group.

1.3.1 The Involvement of Extra-Hepatic Tissues in the Adrenal Control of Carbohydrate Metabolism.

The above observations were among the first to demonstrate how the adrenal cortical hormones promote carbohydrate conservation

at the expense of tissue protein or related non-nitrogenous precursors such as lactate. The questions arise of whether glucose synthesis is stimulated or its utilisation decreased, (or both), and of which tissues are implicated in these events.

Selye and Dosne (1940) demonstrated that adrenal cortical extract could inhibit the reduction in blood glucose level following hepatectomy in fasted rats. Similarly, Reinecke (1943) reported reduced plasma glucose levels in eviscerated rats following adrenalectomy, thus implicating extra-hepatic tissues in the adrenal control of circulating glucose levels. Several workers have demonstrated that the level of plasma amino acids in the eviscerated rat is reduced following adrenalectomy and restored to normal by cortisol administration.This observation shows the importance of adrenal hormones in maintaining the release from extra- hepatic tissues of amino acids which may subsequently serve as substrates for hepatic gluconeogenesis. (Ingle et. al. 1948; Bondy, 1949; Tilton et. al. 1955).

Ingle (1941) demonstrated that in rats made markedly hyperglycaemic with cortisone injections over several days, glycosuria was in excess of the amount of glucose synthesis attributable to protein catabolism as measured by nitrogen excretion. Long et. al. (1960) detected no increase in urea excretion during the first few hours following steroid administration; thus a source other than protein for the carbon atoms of glucose formed during this period seems likely.

1.3.2 The Effects of Adrenal Corticosteroids upon Basal Hepatic Gluconeogenesis.

Following the work of Lewis et. al. (1940) concerning gluconeogenesis from lactate, Winternitz et. al. (1957) demonstrated that the quantities of liver glycogen formed after the administration of glucose, fructose, glycerol, lactate and malate to adrenalectomized rats were increased following cortisol treatment. Of particular note was the observation that only minimal glycogen formation occurred from lactate unless cortisol was administered.

Koepf et. al. (1941) observed diminished carbohydrate formation in liver slices incubated with lactate and pyruvate when the slices were prepared from adrenalectomized rats. Eisenstein et. al. (1964) reported that dexamethasone (in vitro) increased gluconeogenesis from alanine but not from pyruvate in liver slices prepared from normal or adrenalectomized rats.

Landau et. al. detected no increase in the incorporation of ^{14}C into glucose and glycogen from 40mM 2-(^{14}C)-pyruvate or $^{14}CO_2$ in cortisone treated liver slices from normal rats. However, when the concentration of pyruvate in the incubation medium was reduced to 0.6 mM, incorporation of ^{14}C was increased two-fold following incubation with cortisone. Uete and Ashmore (1963) failed to observe increased incorporation of ^{14}C from (^{14}C)-glycerol into glucose and glycogen in rat liver slices treated with cortisol, and thus suggested that steroids stimulate a reaction between alanine and dihydroxyacetone phosphate.

Miller (1961) observed no change in urea production

or glucose release in response to cortisol treatment in the perfused livers of fasted rats. An enhancement, by cortisol, of the ability of glucogon to increase hepatic urea production was observed.

Exton and Park (1965) perfused livers from normal and adrenalectomized rats in the presence of lactate and fructose, and measured glucose production and the concentration of glycolytic intermediates. They reported reduced gluconeogenesis from lactate in the livers of adrenalectomized rats due to inhibition of the conversion of pyruvate to triose phosphates. Results of studies on pyruvate carboxylase and phosphoenolpyruvate carboxykinase activity support this contention (Henning et. al. 1963; Shrago et. al. 1963). In contrast to these results, Eisenstein et. al. (1966) found that gluconeogenesis from lactate was similar in the livers of adrenalectomized and normal rats perfused in the absence of red blood cells. In similar experiments, gluconeogenesis from alanine was impaired in the perfused livers of adrenalectomized rats and could be restored to normal by dexamethasone treatment in vivo or in vitro. These results are similar to those obtained in vivo by B. Friedmann et. al. (1965) and suggest that gluconeogenesis from three carbon precursors is not normally dependent upon adrenal corticosteroids.

Haft et. al. (1972) pointed out that impaired gluconeogenesis from alanine and lactate in the perfused liver of the adrenalectomized rat has only been demonstrated in the presence of large concentrations of substrates where the maximum capacity of the liver to convert the substrate to glucose is tested. In the presence of more physiological concentrations of alanine,

adrenalectomy did not reduce the ability of the perfused liver to convert this substrate to glucose. Evidence supporting this contention was reported by Friedmann et. al. (1965), who observed no decrease in the conversion of 1.0 m mole of alanine to glucose in intact adrenalectomized rats.

1.3.3 The"Permissive" Effect of Adrenal Corticosteroids upon the Hormonal Control of Hepatic Gluconeogenesis.

Whilst the effects of glucocorticoids upon basal hepatic gluconeogenesis remain unclear, there is more general agreement concerning the "permissive" effect of steroids upon hormone-stimulated glucose production. Ingle (1952) was the first person to suggest that glucocorticoids might act by "permitting" other hormones to exert their effects.

Levy-Simpson (1932) first reported that patients with Addison's disease failed to exhibit hyperglycaemia following injection of adrenalin. Thorn et. al. (1940) confirmed this result and found that steroid treatment restored the glycaemic response to the usual level.

Exton and Park(1966) reported that glucagon did not exert its normal stimulatory effect upon gluconeogenesis from lactate in the perfused livers of adrenalectomized rats. N. Friedmann et. al. (1967) confirmed these results and showed that dexamethasone treatment in vivo or in vitro restored the gluconeogenic response to glucagon. Dexamethasone alone produced no increase in gluconeogenesis. N. Friedmann et. al. (1968) extended the above

observations by reporting that in addition to glucagon-stimulated gluconeogenesis, the adrenalin-stimulated process was abolished in the perfused livers of fasted adrenalectomized rats. These workers found that the normal hormonal responses were rapidly restored by treatment with dexamethasone or cortisol in vivo or in vitro, while the steroids alone exerted little effect upon basal gluconeogenesis under these conditions. The failure of the livers from fasted adrenalectomized rats to respond to these hormones was not due to diminished production of hepatic cyclic AMP, since the addition of exogenous cyclic AMP to the perfusion medium similarly failed to stimulate hepatic gluconeogenesis. On the basis of these results, N. Friedmann et. al. (1968) suggested that the adrenalectomized rat has a reduced sensitivity to cyclic AMP with regard to the stimulation of hepatic gluconeogenesis.

Adrenal steroids also exert a permissive effect upon some of the changes which occur in carbohydrate metabolism following the onset of diabetes. Exton et. al. (1973) have recently reported that adrenalectomy reduces, and steroid treatment restores, gluconeogenesis from lactate in the perfused livers of alloxan diabetic rats. These workers also found that adrenal ablation diminished the increase in phosphoenol pyruvate carboxykinase activity in such livers, thus confirming the earlier work of Shrago et. al. (1963) in intact rats. Subsequent cortisol treatment for one hour restored the enzyme to its previous level.

It is clear that glucocorticoids can exert powerful permissive effects during the elevation of gluconeogenesis caused by a number of hormonal stimuli. In the present study, the influence of adrenalectomy upon vasopressin-stimulated gluconeogenesis in the

perfused liver (Hems and Whitton, 1973) has been investigated.

1.3.4 The Effects of Adrenal Corticosteroids upon Hepatic Glycogen Metabolism.

Since the classical observation of Britton and Silvette (1932, 1934) that bilateral adrenalectomy in the rat and the cat depleted carbohydrate reserves in liver and muscle and caused hypoglycaemia, there has been abundant evidence that adrenal glucocorticoids exert a profound effect upon hepatic glycogen metabolism. Katzin and Long (1938) observed a marked increase in the liver glycogen content of fasted rats and mice after intra-peritoneal injections of cortical extract. There are various reports in the literature indicating that the adrenalectomized rat is unable to synthesize liver glycogen from glucose, lactate, pyruvate and alanine at the normal rates,(Bluell et. al. 1936; Evans,1941; Holmes,1940; Wong,1950). Liver slices prepared from adrenalectomized rats have been shown to exhibit a similar defect in glycogen synthesis, (Bendall and Lehmann,1942; Lipsett and Moore,1951). Adrenal cortical extract has been reported to increase glycogen synthesis from these precursors in liver slices prepared from both normal and adrenalectomized rats, (Koepf et. al. 1941; Chiu and Needham,1950; Seckel,1940).

Corey and Britton (1941) have reported that in the perfused rat liver adrenal cortical extract prevents glycogenolysis, while in the perfused cat liver glycogen synthesis itself is increased within 15 minutes of addition of the extract in vitro.

More recent studies in intact rats (B.Friedmann et. al. 1967) have shown that the starved re-fed adrenalectomized rat will synthesize liver glycogen at normal rates after an initial lag phase of 2 hours. However, while the initial rate of synthesis was normal in these animals, the level of hepatic glycogen did not reach the maximum values found in normal rats. Adrenalectomized rats treated with cortisol for 3 days responded in the normal fashion.

Extensive research on the enzymes of glycogen synthesis supports the general view that adrenal corticosteroids act to promote this process in the liver. Thus, Hilz et.al. (1963) have shown that the activity of glycogen synthetase was increased two-fold six hours after a single cortisol injection in adrenalectomized rats. Glycogen synthesis itself, however, was stimulated within 2 hours and Hilz suggested that this early stimulation of synthesis was due to activation of the "b" form of glycogen synthetase by increased intracellular levels of glucose-6-phosphate. In a similar study, Hornbrook et. al. (1966) demonstrated that adrenalectomy does not lower the total amount of glycogen synthetase in the rat liver, but rather converts the enzyme to the glucose-6-phosphate dependent "b" form. These workers found that cortisol treatment of fasted adrenalectomized rats reversed these changes within 3 hours, but in contrast to the results of Hilz et.al(1963), glucose-6-phosphate levels did not rise.

Not only do steroids influence glycogen synthesis in the liver, these hormones also appear to play a permissive role in hormonally induced hepatic glycogen breakdown. Schaeffer et. al. (1969) found that the hyperglycaemic effect of adrenalin or

cyclic AMP in vivo was diminished following adrenalectomy, although the level of active glycogen phosphorylase was not affected. However, the levels of the inactive enzyme were very much reduced following adrenalectomy, and adrenalin or cyclic AMP administration did not increase the activity of the available enzyme. Cortisol treatment for 3 days restored normal inactive enzyme levels and the normal glycaemic response to adrenalin and cyclic AMP. In another study in vivo, Issekutz and Borkow (1973) showed that 3 days treatment with methyl prednisolone (3 mg kg^{-1} day^{-1}) strikingly potentiated the effect of glucagon upon hepatic glucose output and plasma glucose concentration in unanaesthetised dogs with indwelling catheters.

Exton et. al. (1972a)have reported that high concentrations of adrenalin, glucagon or cyclic AMP stimulate both glycogenolysis and gluconeogenesis in the perfused livers of fed adrenalectomized rats. However physiological levels of the hormones or cyclic AMP did not stimulate these processes or increase phosphorylase activity in such livers, thus indicating reduced sensitivity to cyclic AMP.

In the present study, the hepatic glycogenolytic response to the hormone vasopressin (Hems and Whitton 1973), has been studied in the perfused liver. Experiments are described in which the role of the adrenal gland in maintaining this response is characterised.

1.4 The Influence of Vasopressin upon Intermediary Metabolism.

Borchardt (1908) first reported that the injection of an extract of the posterior hypophysis produced hyperglycaemia and glycosuria in rabbits. Imrie (1929) suggested that the extra glucose was derived from hepatic glycogen. Since these early results, hyperglycaemia has been reported by numerous workers to result from treatment with posterior pituitary extracts (see Mirsky, 1968 for references).

With the advent of pure preparations of posterior pituitary hormones, vasopressin was reported to exert a number of metabolic effects in mammals. Thus, high concentrations of the hormone have been shown to induce a prompt hyperglycaemic response in the intact dog (Bergen et. al. 1960) and in rat liver slices (Heidenreich et. al. 1962, 1963). In the latter study, the rise in blood glucose concentration was associated with a decline in hepatic glycogen content. Vaisler et. al. (1965) demonstrated that vasopressin can stimulate the release of glucose from the perfused liver, and Hems and Whitton (1973) reported that hepatic glucose release and gluconeogenesis in the perfused rat liver were stimulated by physiological concentrations of the hormone.

The rapid intravenous injection of vasopressin has been reported to decrease plasma free fatty acid concentrations in dogs (Mirsky 1962) and rats (Itoh et. al. 1966, Hems et. al. 1975b). Very recently, vasopressin has been reported to inhibit fatty acid synthesis in the perfused mouse liver (Ma and Hems 1975).

In the present work the characteristics of the metabolic actions of vasopressin have been studied in the perfused rat liver, and an attempt has been made to elucidate the mechanism of these hormone actions.

1.5 The Mechanism of Short Term Hormone Actions in the Liver.

It is possible to classify hormones into two classes on the basis of the mechanisms by which they affect the target tissues. Thus, one class contains those hormones which act on the cell surface such as catecholamines and peptides and the other those hormones such as steroids and thyroxine which actually enter the cell.

Rall et.al.(1956)first showed that the hormone adrenalin resulted in the production of a heat stable dialysable factor in the canine liver which was able to activate the enzyme glycogen phosphorylase "b". This factor was later shown to be adenosine -3', 5'- cyclic monophosphate (cyclic AMP) (Sutherland and Rall 1958;Lipkin et. al. 1959), a nucleotide which is now known to be ubiquitous in living organisms. Sutherland and his co-workers have termed cyclic AMP the "second messenger", the first messenger being the hormone which increases the activity of adenylate cyclase, the enzyme which catalyses cyclic AMP formation (Robison et. al. 1971).

Since the first demonstration of the involvement of cyclic AMP in hormone action, many short term hormonal effects have been shown to be mediated by the nucleotide. These include several short-term hormone effects upon hepatic metabolism (Robison et. al.1971). In these effects, cyclic AMP appears to have the ability to activate inactive protein kinases within the cell, thus rendering them able to phosphorylate, and thus activate other inactive enzymes (Walsh et. al. 1968). Kuo and Greengard (1969) have found cyclic AMP dependent protein kinases from about thirty sources, including

mammalian and invertebrate tissues.

However, it is clear that not all short term hormone actions in the mammal are mediated by cyclic AMP. Insulin, for example, has been shown to increase the activity of glycogen synthetase in muscle without affecting cyclic AMP levels (Craig et. al. 1969). There have been recent reports which suggest that the classical effect of adrenalin in raising the hepatic concentration of cyclic AMP may not have the physiological significance which has been attributed to it in the past (Tolbert et. al. 1973).

As evidence has accrued suggesting that cyclic AMP is not the universal mechanism by which all short term hormonal effects occur, considerable interest has been shown in other "second messenger" molecules. Thus, guanosine -3', 5'- cyclic monophosphate (cyclic GMP) has been shown to be present in very many mammalian tissues although usually in much lower concentrations than cyclic AMP (Goldberg et. al. 1969). The hepatic concentration of cyclic GMP has been shown to be influenced by several hormones including secretin, adrenal steroids, (Thompson et. al. 1973) and insulin (Shaw et. al. 1972).

Another series of compounds which have been studied in relation to a possible role as mediators of hormone action are the prostaglandins. Prostaglandins have been shown to reduce cyclic AMP levels in adipose tissue (Butcher and Baird 1968). Since lipolytic hormones (which are thought to act via increased levels of cyclic AMP) increase the rate of efflux of prostaglandins from rat fat pads in vitro, (Shaw and Ramwell 1968) it is possible that these compounds may act as a physiological feedback control mechanism in adipose tissue.

There is growing evidence that some short term actions of hormones upon the liver are cation dependent. The stimulation, by glucagon and adrenalin, of gluconeogenesis from lactate has been shown to be critically dependent upon Ca^{++} and K^{+} concentrations (Tolbert and Fain 1974; Pilkis et. al. 1975). Ca^{++} deficiency does not influence the accumulation of cyclic AMP in response to these hormones, so it seems likely that cyclic AMP requires Ca^{++} in order to influence hepatic gluconeogenesis.

In the present study, the short term effects of the hormone 8-arginine vasopressin upon hepatic metabolism have been studied in the perfused rat liver. Vasopressin exerts many glucagon- or catecholamine-like effects in the rat liver, and experiments have been devised to investigate the mechanism of action of this hormone and any similarities with the modes of action of glucagon and adrenalin.

1.6 Scope and Aims of the Present Study.

The considerations described above indicate that many aspects of the control by adrenal steroids of hepatic carbohydrate and lipid metabolism require further clarification. Similarly, whilst the enormous volume of recent work concerning the mechanism by which hormones exert their short term metabolic effects has done much to increase our understanding of this subject, it seems clear that all such hepatic effects are not solely the result of increased intracellular cyclic AMP concentrations.

1.6.1 The Perfused Rat Liver as a Model for the Study of the Hormonal Control of Hepatic Metabolism.

Any study of the metabolism of a particular organ may involve experiments in vivo or in vitro. In vivo studies of organ function involve the difficulty that direct effects of hormones, drugs etc. are hard to establish. This is because changes in one tissue often result in similar or compensatory changes in other tissues, and nervous or hormonal responses which may profoundly influence the particular organ under investigation.

In vitro studies of hepatic metabolism have generally involved the following techniques :-

(1) Homogenates

(2) Liver slices

(3) Isolated liver cells (hepotocytes)

(4) Liver perfusion

Homogenates have often been used for the study of enzymes and pathways. Liver slices have been used extensively for the study of metabolism. However, in such experiments many cell constituents leak out into the incubation medium. Hence studies concerning metabolic control are best not performed with such slices. Hepotocytes have been used extensively in recent work and most metabolic processes such as gluconeogenesis (Johnson et. al. 1972) and lipogenesis (Goodridge 1973) appear to be preserved. However, hepotocytes have been reported to be insensitive to some hormones and other metabolic regulators (Garrison and Haynes 1973), although this problem is being resolved. Hepotocyte preparations

involve only one cell type, non-parenchymal cells not usually being isolated. Also, since the normal blood circulation of the liver is not present in such preparations some metabolic effects which are found in vivo may be absent in hepotocytes (and vice-versa).

In view of the above considerations, the isolated perfused rat liver was selected as being suitable for most of the experiments in the present study. The perfused liver involves only minor disruptive procedures during its preparation, which may be performed under anaesthetic, and the preparation exhibits superior biosynthetic rates as compared with most other liver preparations (Windmueller and Spaeth 1966). Since the volume of the perfusion medium can be made very large in relation to liver size, a high degree of control may be exerted over the composition of this medium. Hence, a perfusion medium of known composition may be employed, which is not rate limiting to basic hepatic functions such as oxygen consumption or nutrient uptake. Similarly, optimum concentrations of circulating substrates of constant specific radioactivity can be maintained in the perfusate. Finally, liver perfusion offers the possibility of studying the effects of circulating hormones upon liver metabolism and, in conjunction with experiments in vivo, of assessing whether such effects upon the liver are of a direct or indirect nature.

1.6.2 The Specific Aims of the Experiments Described in the Present Study.

The previous sections reveal the extent of the confusion surrounding the role of the adrenal gland in the control of lipid and carbohydrate metabolism in mammals.

In the present study, experiments were designed to elucidate the role of adrenal corticosteroids in the control of hepatic and extra-hepatic lipogenesis in both normal and diabetic rats. In many experiments, hepatic cholesterol synthesis was studied in parallel with fatty acid synthesis, esterification and release. Thin layer chromatography was employed in order to separate lipid classes, and also fatty acids according to their degree of unsaturation. Thus the detailed effects of adrenalectomy and steroid replacement upon lipogenesis could be assessed.

Most previous measurements of fatty acid synthesis have been made using ^{14}C-labelled precursors. Such measurements do not necessarily represent total fatty acid synthesis (Hems et. al. 1975), and in the present study, the incorporation of ^{3}H from $^{3}H_2O$ has been used as a measure of total fatty acid synthesis de novo (Windmueller and Spaeth, 1966). The simultaneous use of ^{14}C-labelled precursors has permitted the determination of the proportional contribution of these precursors to the total rates of synthesis.

Since lipogenesis consumes carbohydrate precursors, it is possible that adrenal corticosteroids could influence this process in the animal by the direct control of carbohydrate metabolism. The role of these hormones in permitting other hormones to exert their physiological effects upon carbohydrate metabolism is well

established. In the present study, the steroid dependence of vasopressin-stimulated glycogenolysis and gluconeogenesis (Hems and Whitton, 1973) has been studied with a view to further clarifying the role of the adrenal gland in the control of hepatic glucose mobilisation.

Recent studies have demonstrated the influence of vasopressin upon hepatic carbohydrate and lipid metabolism (Hems and Whitton, 1973; Ma and Hems, 1975; Hems et. al. 1975). In the present study, the hepatic effects of this hormone were further characterised, and experiments were designed to elucidate it's mechanism of action. The sensitivity of vasopressin-stimulated glycogenolysis to insulin, (which has been reported to antagonise the glucagon-stimulated process :Glinsmann and Mortimore, 1968), was studied in the perfused liver. In order to obtain detailed information concerning the effects of vasopressin upon hepatic metabolism, the intracellular concentrations of various metabolic intermediates were measured before and after hormone treatment and compared with the published effects of other hormones. Partly as a response to the results of these studies, and in view of the importance of carbohydrate precursors in hepatic lipid metabolism, the direct effects of vasopressin upon hepatic fatty acid synthesis and ketogenesis were investigated in the present study.

Finally, since vasopressin influences hepatic carbohydrate metabolism in a similar fashion to glucagon and adrenalin, experiments were designed to assess the roles of cyclic nucleotides and cations in the hepatic mechanism of vasopressin action. The role of extracellular Ca^{++} ion concentration was studied, since the control of hepatic gluconeogenesis by adrenalin and glucagon has been reported to be Ca^{++} dependent (Tolbert and Fain,1974;Pilkis et.al.1975).

2 Animals, Materials and Methods.

2.1 Animals.

2.2 Sources of Materials and Equipment.

2.3 The Preparation of Fatty Acid Substrates for Liver Perfusion.

2.3.1 The Preparation of Fat Free Albumin.

2.3.2 The Preparation of Stock Solutions of Albumin-Bound Fatty Acids.

2.4 The Perfusion of the Isolated Rat Liver.

2.4.1 The Perfusion Apparatus.

2.4.2 The Composition of the Perfusion Medium.

2.4.3 Surgical Operating Procedure.

2.4.4 Sampling During the Course of Perfusion.

2.4.5 The Collection and Assay of $^{14}CO_2$ Released by the Perfused Liver.

2.5 Experiments in Vivo.

2.5.1 The Measurement of Fatty Acid Synthesis.

2.5.2 The Measurement of Net Glycogen Accumulation.

2.5.3 The Measurement of Various Hormonal Effects upon Intracellular Adenosine-3',5'-Cyclic Monophosphate Concentration.

2.6 Separation Techniques.

2.6.1 The Extraction and Isolation of Lipids from Tissues and Perfusate.

2.6.2 The Separation of Lipid Classes by Thin Layer Chromatography.

2.6.3 The Separation of Fatty Acids According to their Degree of Unsaturation by Thin Layer Chromatography.

2.7 Analytical Methods.

2.7.1 The Assay of Free Fatty Acids in Saponified Perfusion Medium.

2.7.2 The Estimation of Circulating Glucose and Hepatic Glycogen.

2.7.3 The Determination of Glycolytic and Other Intermediates Involved in Carbohydrate Metabolism.

2.7.4 The Assay of Cyclic Nucleotides in Liver and Perfusate.

2.7.5 Liquid Scintillation Spectrometry.

2.8 The Calculation of Rates of de novo Synthesis of Fatty Acids and Cholesterol from the Incorporation of ^{3}H from $^{3}H_2O$ and ^{14}C from ^{14}C-Precursors.

2. Animals, Materials and Methods.

2.1 Animals

The albino Sprague-Dawley rats used in the present study were of the CFX strain from Carworth. The rats were bred in the Biochemistry Department at the Imperial College, the male breeding stock being renewed every three months. They were maintained on a light/dark cycle of 12 hours (light from 06.00 h. - 18.00 h. GMT) at constant relative humidity and temperature (19 - 23°C)

The animals used in the work described here were fed Thompsons rat cake (Pilsbury's Ltd. Birmingham and Heygate and Sons, Northants) ad libitum. For experiments involving the use of starved rats, food was withdrawn for 48 hours prior to experimentation from 10.00 hrs.

Rats weighing about 160 gms. were bilaterally adrenalectomized, or sham-operated, under diethyl-ether anaesthesia 7 - 12 days prior to use. The adrenal glands were exposed by means of twin incisions, each about 1 cm. long on either side of the spinal column just posterior to the rib cage. Each gland and the surrounding adipose tissue was teased away from the kidney and excised with a small pair of scissors. In sham operations the gland was merely exposed to view and then replaced in position in the abdominal cavity. It was not found to be necessary to ligature the surenal artery and vein. Two ligatures (Surgical Linen Suture Thread size 2/0, Industria Britanica, U.K.) were placed in each incision and tied off. The incisions in the epidermis were closed with 11 mm. suture clips (John Weiss and Son, London, U.K.) If, after an adrenalectomy had been performed, any doubt existed as

to the complete removal of both adrenal glands , the animal in question was not used for subsequent experiments. After the operation the rats were maintained on 0.9% (w/v) sodium chloride in their drinking water and weighed daily at 10.00 hr. Growth curves were constructed for both sham operated and adrenalectomized rats at two seasons of the year and these appear in figures 1 and 2. The food intake of adrenalectomized and sham operated rats was measured by weighing the food remaining each day for pairs of animals kept in a single cage for 12 days following operation. Food intake curves constructed from this data appear in figure 3. It is clear that between 3 and 7 days after surgery, sham operated and adrenalectomized rats (both maintained on 0.9% Na Cl) gain weight at a similar rate and consume similar amounts of food. This observation is in agreement with the results of Edwards (1973).

When adrenalectomized rats were used in subsequent experiments their carcasses were always checked for complete removal of the adrenal glands at the end of the experiment. The results from any animals which exhibited visible traces of the gland were discarded.

When required, rats (weighing about 210 gm.) were made diabetic by the injection of 75 mg./kg. streptozotocin (dissolved in 0.25 ml. 0.01 M Na-citrate buffer pH 4.5.) into a tail vein of a conscious animal. The initial weight of the rat was noted and the animals weighed daily thereafter. Growth curves for the diabetic rats are to be found in figure 4. Four days after the injection of streptozotocin the blood glucose concentration of the animal was determined in a sample withdrawn from a tail vein with a heparinised syringe. Animals which exhibited elevated plasma

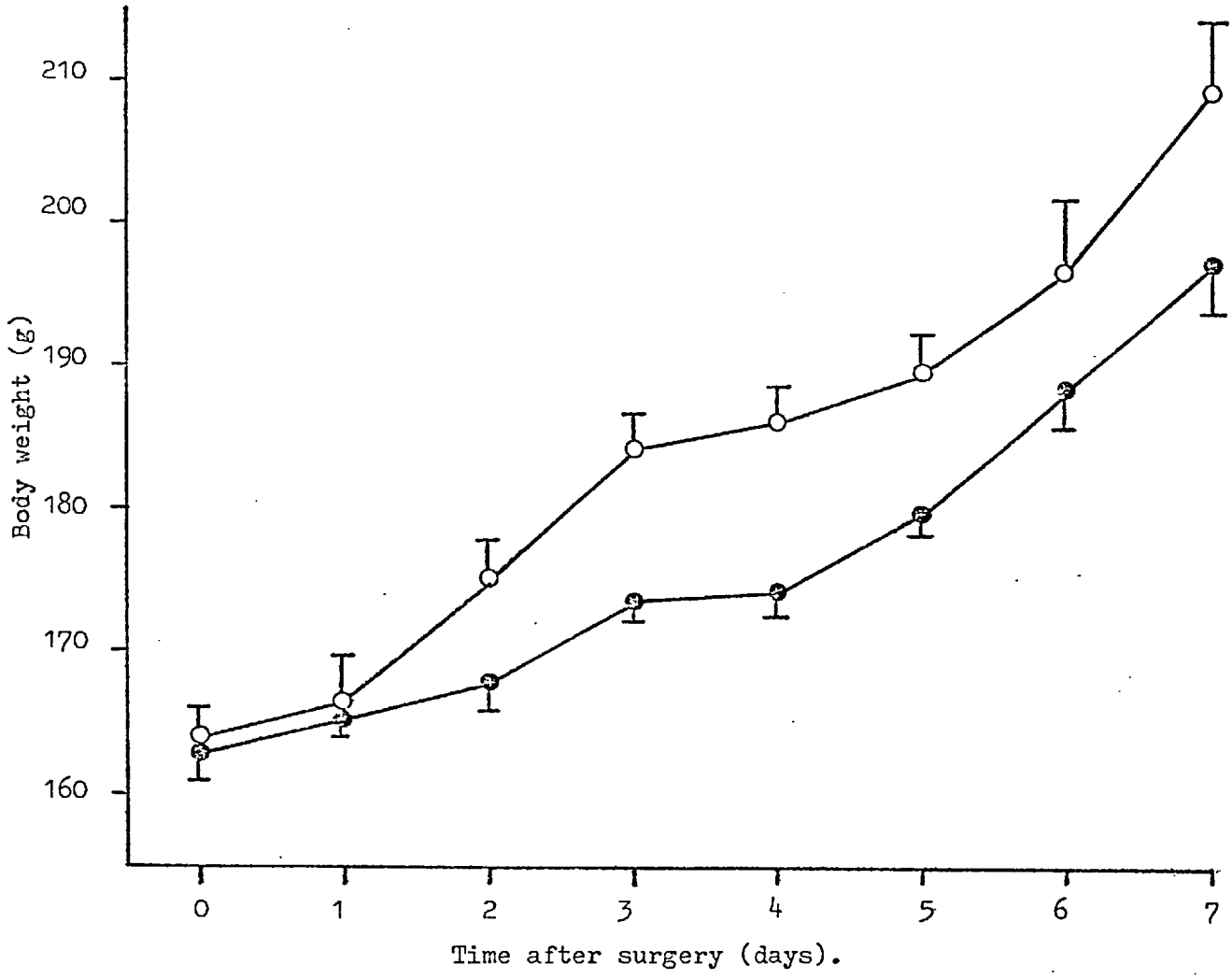


Fig. 1 Growth curves of adrenalectomized and sham-operated rats in January.

Rats were bilaterally adrenalectomized (●) or sham-operated (O) on day zero as described in the text. They were weighed at the time of surgery and at a similar time on each of the following 7 days. Each point is the mean ± S.E.M. of 9 animals in each group.

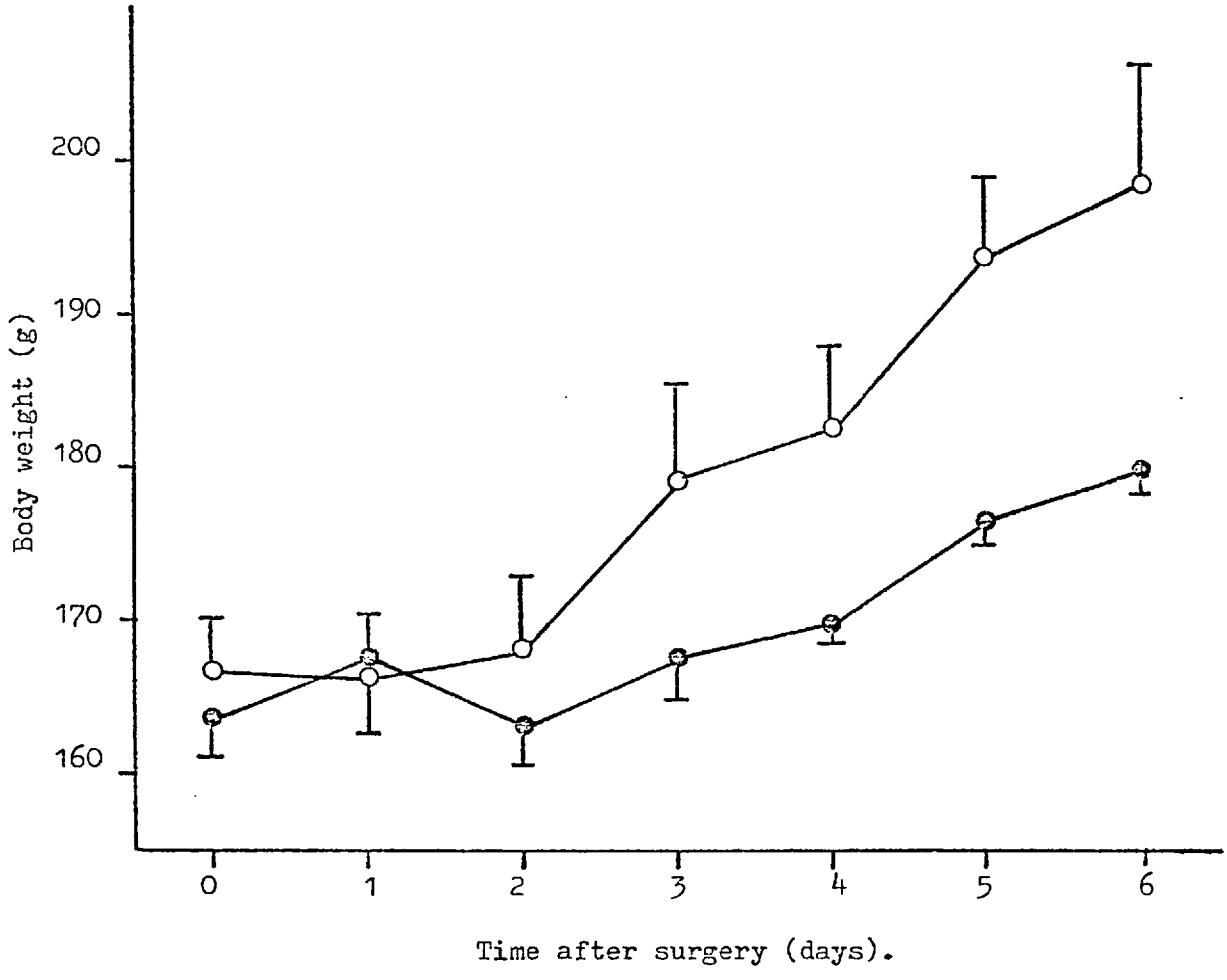


Fig. 2 Growth curves of adrenalectomized and sham-operated rats in June.

Rats were bilaterally adrenalectomized (●) or sham-operated (O) on day zero as described in the text. They were weighed at the time of surgery and at a similar time on each of the following 6 days. Each point is the mean ± S.E.M. of 15 adrenalectomized and 6 sham-operated animals.

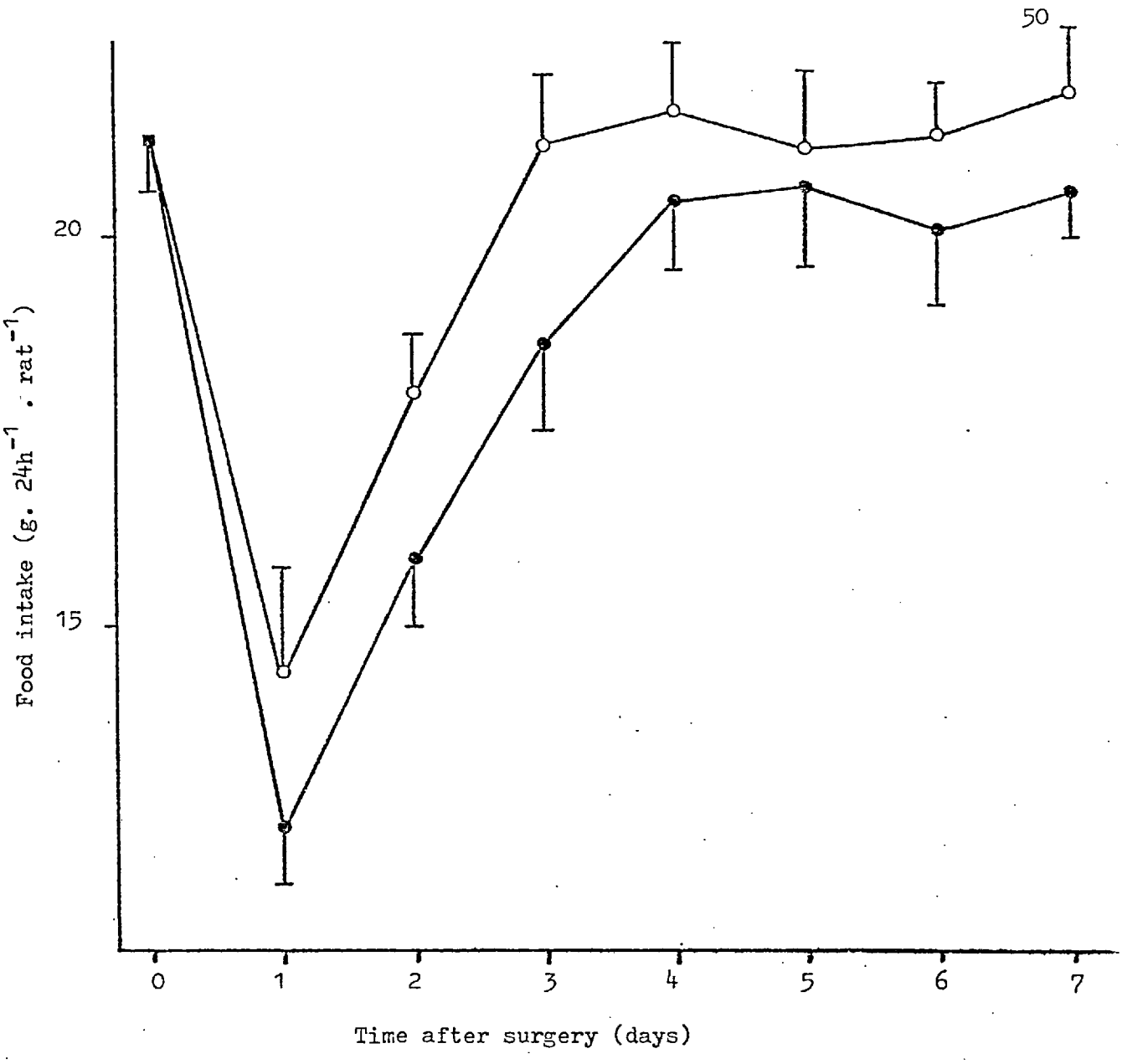


Fig. 3 Food intake of rats after adrenalectomy or sham-operation.

Pairs of male Sprague-dawley rats weighing 160 - 170 gms. were housed in cages measuring 18x23x32 cms. and containing 500 gms. of Thompson's rat cake. On day zero, the rats were adrenalectomized (●) or sham-operated (O) as described in the text. The daily food intake of each pair of similarly operated rats was measured by weighing the amount of food remaining in each cage at 24 hourly intervals. Results are means $\pm$ S.E.M. of the data from 3 pairs of rats.

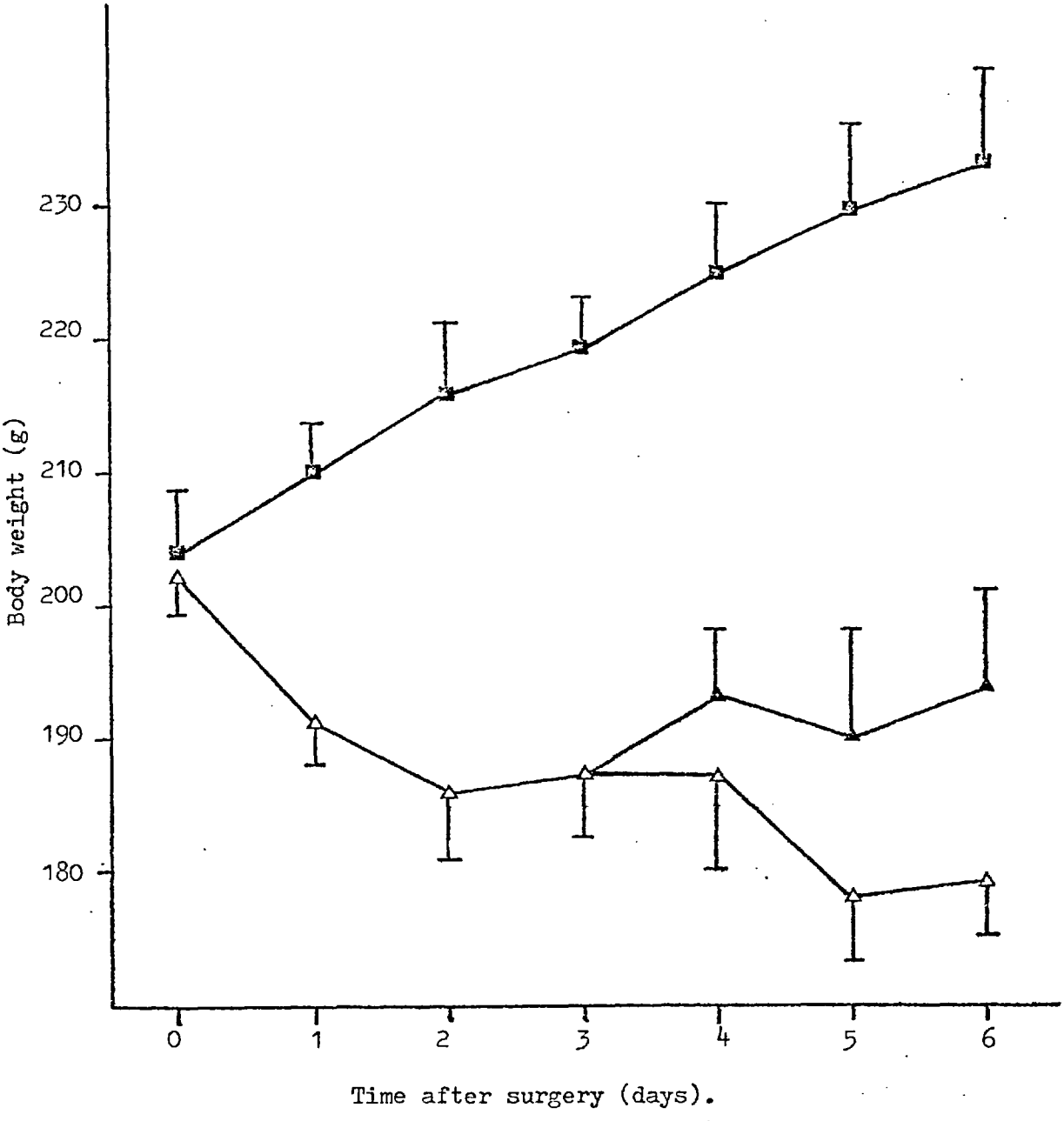


Fig. 4 Growth curve of streptozotocin diabetic and citrate injected control rats in July.

Rats were injected with streptozotocin (75 mg./kg.) in 0.01 M citrate buffer (pH 4.5) (Δ) or with citrate buffer alone (■) on day zero as described in the text. On day 3 some of the diabetic rats were adrenalectomized (▲). The rats were weighed at the time of injection and at a similar time on each of the following 6 days. Results are means ± S.E.M. of 5 animals in each group.

glucose concentrations (> 12 mM) and an overall loss of body weight over this period were used in experiments. Some animals were injected with 0.01 M Na-citrate buffer (pH 4.5) alone to serve as controls for the experiments involving diabetic rats. All experiments involving the use of diabetic rats were performed between 7 and 12 days of the injection of streptozotocin.

2.2 Sources of Materials and Equipment.

All solvents and reagents were of AnalaR grade and were obtained from Hopkins and Williams (Chadwell Heath, Essex, U.K.), B.D.H. Chemicals Ltd. (Poole, Dorset, U.K.) or Fisons Ltd. (Loughborough, Leicestershire, U.K.) unless otherwise stated. Alcohols were from James Burrough Ltd. (London S.E. 11. U.K.) L-lactic acid, oleic Acid, palmitic Acid, Trizma base and the hormones hydrocortisone-21-sodium succinate, 8-arginine vasopressin and adrenalin bitartrate were purchased from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Enzymes and substrates for analytical purposes, nucleotides and pyruvic acid (monosodium salt) were from C. F. Boehringer Corporation (London) Ltd. All radiochemicals were from the Radiochemical Centre (Amersham, Bucks, U.K.). Bovine serum albumin (Pentex, Fraction V) was from Miles Laboratories (Kankakee, Illinois, U.S.A.). Insulin was the highest grade commercial ox preparation from Burroughs Wellcome (Dartford, Kent, U.K.) and glucagon was obtained from Eli Lilly (Indianapolis, U.S.A.). Streptozotocin was prepared and donated by Dr. E. Karunanayke (Imperial College, London).

The anaesthetic 'Nembutal' was obtained from Abbot Laboratories Ltd. (Queenborough, Kent, U.K.) and heparin was from Evans Medial Supplies (Liverpool, U.K.). Canulae and other surgical apparatus were from Holborn Surgical Instrument Co. (London W.C.1., U.K.). Silicone rubber tubing (Silescol) was from Esco (Rubber) Ltd. (London W.1., U.K.) and plastic tubing was from Portland Plastics Ltd. (Hythe, Kent, U.K.).

The(8-arginine)-vasopressin from Sigma (London) Chemical Co., was prepared from synthetic vasopressin of about 360 units/mg. activity. The activity of each batch used was checked by bio-assay of its antidiuretic effect in the ethanol loaded rat (assays kindly performed by Dr. M. Forsling, Dept. of Physiology, Middlesex Hospital) and generally found to be about 80% of the stated activity. All doses of the hormone were calculated on the basis of this measured activity.

Water soluble materials were added to perfusions in 0.9% Na Cl or water (if the added volume was negligable) unless otherwise stated. Where appropriate, such materials were neutralised with NaOH or HCl.

2.3 The Preparation of Fatty Acid Substrates for Liver Perfusion.

In two series of experiments the liver was perfused with (^{14}C)- labelled fatty acids in the circulating perfusate. These fatty acids were first bound to fat free albumin which was prepared as described below.

2.3.1 The Preparation of Fat Free Albumin.

Bovine serum albumin was de-fatted according to the method of Chen (1967). Thus, 5% (w/v) finely divided activated charcoal (Norit A, Norit - Clydesdale Co. Ltd., Glasgow, U.K.) was mixed with a 10% solution of albumin in distilled water and the pH of the mixture reduced to 3.0 by the addition of 0.2 N HCl. The solution was stirred magnetically for 1 hr. in an ice bath and the charcoal was removed by centrifugation at 20,200 g for 20 mins. at 4°C. The clarified supernatant was then brought to pH 7.0 by the addition of 0.2 N sodium hydroxide and the sodium chloride removed by dialysis against distilled water (four changes in two days). The de-fatted bovine serum albumin was then freeze dried and stored at 4°C until required.

2.3.2 The Preparation of Stock Solutions of Albumin Bound Fatty Acids.

In some experiments albumin bound fatty acids were infused so that their concentration in the perfusate was maintained at 0.7-1.0 mM. For this purpose, a stock solution of 10 mM. fatty acid bound to albumin was prepared according tc the method of Krebs et. al. (1969).

A strong solution (35%) of de-fatted bovine serum albumin was first prepared by slowly adding the freeze dried powder to distilled water with constant stirring. A 20 mM. saponified solution of the fatty acid was then prepared by adding 5 M KOH dropwise to the appropriate amount of the fatty acid

(carrier + ^{14}C labelled) with constant warming on a hot plate. When the fatty acid was just saponified (i.e. when the solution was soapy and clear), the solution was made up to volume with distilled water and kept hot so as to ensure that no fatty acid precipitated. The 20 mM solution of the fatty acyl potassium salt was then added dropwise to a slightly smaller volume of the 35% fat free albumin solution which was constantly stirred on ice in the presence of a pH electrode for the constant monitoring of pH. As the soap solution was added, the pH of the fatty acid - albumin complex slowly rose from about pH 4.0, (the usual pH of 35% de-fatted albumin). Care was taken to ensure that the pH of the complex never rose above pH 9.0 in order to avoid denaturing the albumin. When all the soap solution had been added to the albumin, (or earlier if the pH rose above pH 9.0), the final pH of the complex was adjusted to pH 7.5 with HCl. Finally, the complex was made up to volume with distilled water so that its final concentration was 10 mM. The solutions of albumin - fatty acid complex thus made were stored frozen at -20°C until required.

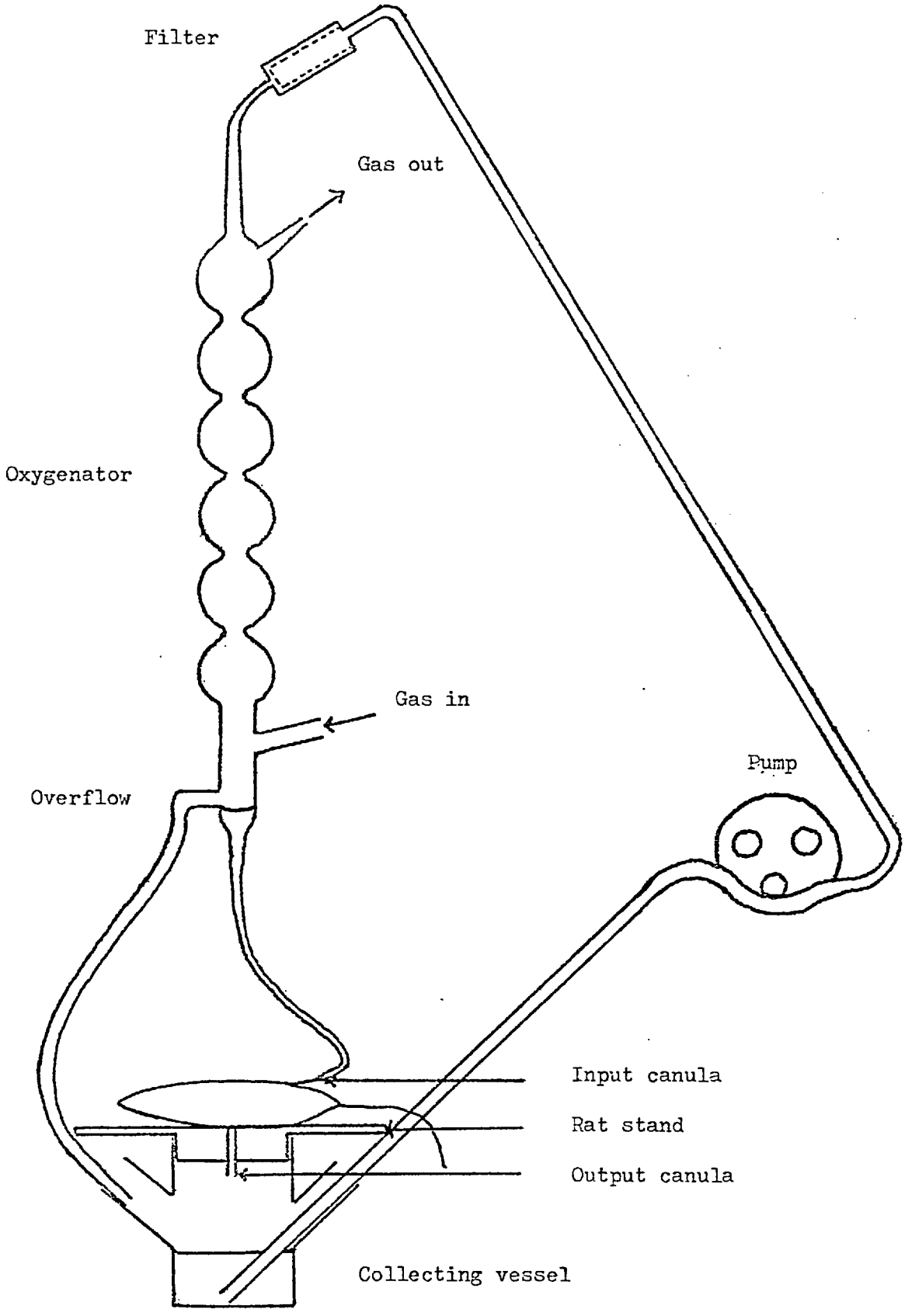
2.4 The Perfusion of the Isolated Rat Liver.

The perfusion technique used in the present study was essentially that described by Hems et. al. (1966) which was based on the method of Miller et. al. (1951).

2.4.1 The Perfusion Apparatus.

The apparatus, arranged as shown in figure 5, was housed in a thermostatically controlled cabinet which permitted perfusion over a variety of temperatures. A sash window in the cabinet facilitated the sampling of liver and perfusate. The perfusion medium, which was contained within a collecting vessel above a magnetic stirrer, was pumped by a MHRE roller pump (Watson Marlow Ltd., Cornwall, U.K.) via a plastic mesh filter to a corrugated glass gas exchanger. Thus a thin film of perfusion medium passed down the inner surface of the gas exchanger which was maintained in a vertical position to ensure efficient gas exchange. A gas mixture of O_2:CO_2 (95:5) (British Oxygen Company) was first saturated with water vapour by passing it through a wash bottle fitted with a sintered-glass distributor. From thence it flowed through the gas exchanger in the opposite direction to the blood flow, thus ensuring maximal oxygenation of the medium, and exchange of CO_2. At the base of the gas exchanger there was a small resevoir of perfusate which was maintained at constant height by means of an overflow tube leading back to the collecting vessel. Thus, by adjusting the position of the gas exchanger in the perfusion cabinet, the hydrostatic pressure of the perfusate entering the liver could be altered. For most experiments, the height of the resevoir above the hepatic portal vein of the experimental animal was maintained at 18 cms. The medium from the constant head resevoir in the gas exchanger was connected to the input cannula of the perfused liver by means of silicone rubber tubing terminating in a male Luer adaptor. The flow through this

Fig. 5 The perfusion apparatus.



tubing was controlled by a rubber clamp. The perfusion medium was collected via a plastic canula in the inferior vena cava and returned to the collecting vessel for recirculation. The arrangement within the perfusion cabinet permitted two sets of apparatus to be operated simultaneously. (Fig. 6).

In some experiments it was necessary to assay metabolites (glucose and cyclic AMP) in the effluent perfusate leaving the liver. For these experiments, the operating tray was removed from above the collecting vessel and supported on a beaker of similar height placed beside the collecting vessel. An extended output tube from the liver passed into the collecting vessel and was secured with tape so that effluent medium could be collected in plastic tubes held in position by means of forceps.

2.4.2 The Composition of the Perfusion Medium.

In most experiments, the standard perfusion medium consisted of 50 mls. bicarbonate buffer (Krebs and Henseleit, 1932) and 10 ml. of 15% (w/v) bovine serum albumin (Pentex Fraction V). The albumin was dialysed for two days against four changes of bicarbonate buffer at 4°C and then kept frozen at -20°C until required. Substrates were added to the perfusion medium as required.

Rat erythrocytes were added to the perfusion medium after 20 mls. of the perfusate described above had been allowed to wash through the perfused liver and discarded. The erythrocytes required were obtained from a fed donor rat whose weight exceeded 600 gms. One animal of this size provided sufficient blood cells

Fig. 6 Two perfused liver preparations in a single cabinet.

For explanation see text.

for a normal perfusion, but occasionally, where a higher volume of perfusate was used, two or more rats might be bled for a single perfusion. The donor rats were bled by aortic puncture under ether anaesthesia using a 20 ml. disposable syringe and a 21 G needle. The blood (16-18 mls.) thus obtained was defibrinated by swirling it with glass beads in a siliconised flask (Baron and Roberts, 1963). Swirling was terminated when a clot appeared in the blood, which was then allowed to stand for 20 mins. while the clot contracted. The supernatant was then divided into two portions and washed twice with 20 volumes of gassed bicarbonate buffer containing the same added substrates as the perfusate. After washing, the pellet was resuspended to a total volume of 10 mls. ready for addition to the rest of the perfusion medium. It was found that 16-18 mls.of whole rat blood from a single donor rat when added to the perfusion medium in this way gave a haematocrit of about 15% at the end of the perfusion period. The pH of this perfusion medium was found to be consistently in the range 7.3 - 7.5 providing that gassing with 5% CO_2 was maintained.

Substrates and hormones were added as described in the next section, sometimes as single doses and sometimes infused at a constant rate(with a delta pump: Watson Marlow Ltd.) so as to maintain the required constant concentration.

2.4.3 Surgical Operating Procedure.

The surgical procedures for liver perfusion used in the present study were essentially those described by Hems et. al.

(1966) as modified by Hems et. al. (1972), except that rats were anaesthetized with diethyl ether. The rat was secured on the operating platform with two strands of adhesive tape. A small beaker containing a diethyl ether soaked pad of cotton wool was placed in position over the animal's head in order to maintain anaesthesia during the operating procedure.

A transverse incision was made across the middle abdomen to expose the gut which was deflected to the animal's left onto a saline soaked tissue. Heparin (200 units in 0.2 ml.) was injected into the posterior vena cava posterior to the renal veins and the haemorrhage thus caused covered with a saline soaked tissue. A ligature (Surgical Linen Suture Thread size 2/0, Industria Britanica, U.K.) was passed around the vena cava anterior to the renal veins and tied loosely. Two ligatures were then tied loosely around the hepatic portal vein so that the most anterior of them included the hepatic artery. The portal vein was then cannulated with a Luer fitting No. 17 Frankis-Evans cannula (trocar and needle) and the needle removed. A rapid backflow of blood when the needle was removed was usually observed, resulting from hepatic artery flow to the liver. If backflow failed to occur, the cannula was carefully filled from a syringe containing saline. When successful cannulation had been achieved the two ligatures around the cannula were tied off thus securing the cannula and occluding the hepatic artery (figure 7).

The thorax of the animal was then opened by a transverse incision about I cm. anterior to and parallel to the diaphragm. A portion of the skin and body wall posterior to this incision was then excised, thus exposing the heart. A ligature was tied loosely

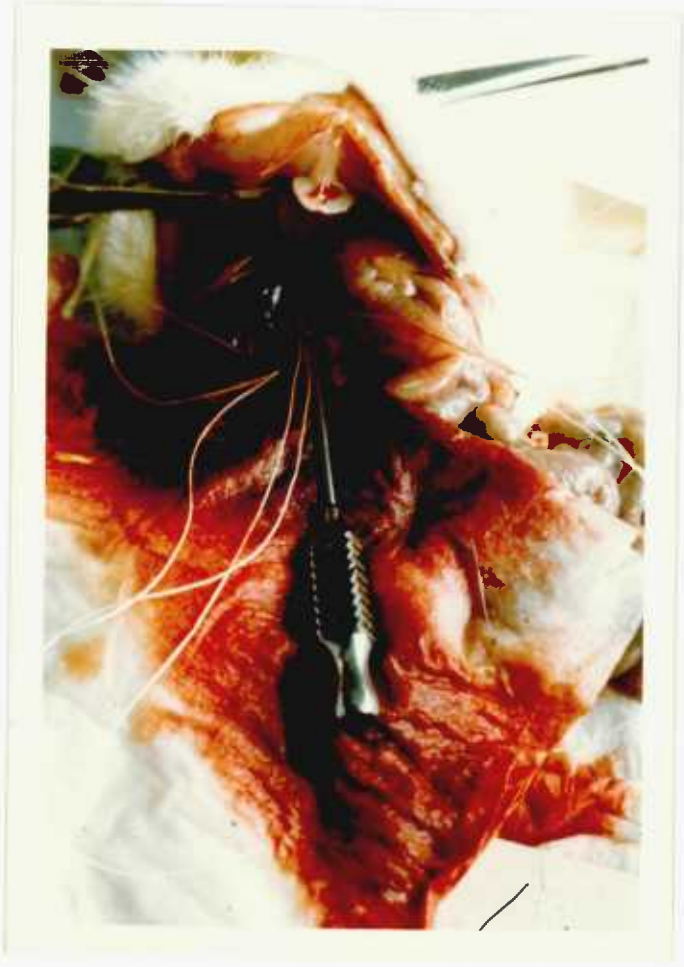


Fig. 7 Successful canulation of the hepatic portal vein prior to the perfusion of the rat liver.

For explanation see text.

around the inferior vena cava and a plastic cannula (constructed of 3 x 2 mm. portex tubing drawn out to a bevelled point) was inserted via the right atrium into the vana cava and down as far as the diaphragm. The ligature was then tightened, thus fixing the cannula in place.

The outflow tube from the bottom of the gas exchanger was then connected to the cannula in the hepatic portal vein and the first 20 mls. of perfusate allowed to flow into a measuring cylinder from whence it was discarded. The flushed out liver was a pale brown colour; if areas of dark red (representing clotting blood) were present then the preparation was discarded.

When the liver had been flushed out in this way, the preparation was placed in position above the collecting vessel and the washed erythrocytes added to the now recirculating medium (figure 8). The entire operative procedure took about 5 minutes, but the time which elapsed between cannulating the hepatic portal vein and washing out the liver was always less then 1 minute. When the preparation had been connected as described above, initial adjustments were made to the roller clamp to give a flow rate of about 17 mls./min. (about 180 drops/minute with the particular tubing employed for outflow to the collecting vessel). The duodenum was then bisected about I cm. below the entry of the bile duct and the portion of the gut anterior to the incision was flushed out with warmed saline from a syringe. This portion of the duodenum was then cannulated with portex tubing (4 mm. x 3 mm.) in order to ensure the free flow of bile during perfusion (this was necessary if the liver was to be maintained in good condition). The volume of bile released by the perfused liver was measured in

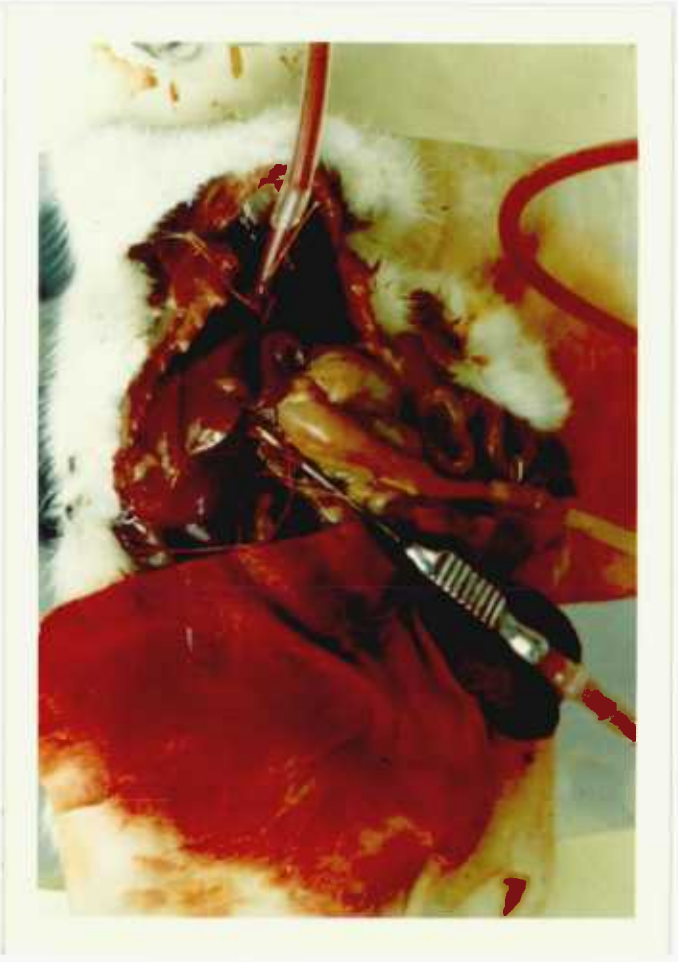


Fig. 8 A successfully completed perfused rat liver preparation.

For explanation see text.

many of the experiments described in the present study; it was typically 0.4 - 1.0 ml./hr., but was not apparently correlated with any of the metabolic processes studied.

When the duodenum had been cannulated as described above, a cage of wire mesh was placed over the liver. This supported a tissue moistened with saline which itself was covered with a polythene sheet to reduce evaporation. The tissue was maintained in a moist condition throughout the perfusion so as to minimise evaporation from the surface of the liver. Final adjustments were then made to the flow rate using the roller clamp. Care was taken to ensure that the temperature inside the cabinet had equilibrated (36 - 36.5°C for most experiments) before the flow rate was adjusted to 16 - 18 ml./min. (180 - 200 drops/min.), since the flow of perfusate through the perfused liver was found to be temperature sensitive.

2.4.4 Sampling During the Course of Perfusion.

Perfusate samples were generally removed from the collecting vessel during the course of perfusion and either spun at 3,000 r.p.m. for 5 minutes in order to remove erythrocytes, or mixed with an equal volume of 6% (w/v) perchloric acid to give an acid extract of whole perfusate. The medium samples were then kept frozen at -20°C. Blood-bourne metabolites (glucose, lactate, cyclic AMP etc.) were measured as described in subsequent sections.

The method of liver biopsy from the perfused liver varied according to the design of the particular experiment. If more

than one liver sample was required from a single perfusion then the first biopsy was made by tying a ligature around the base of the liver lobe to be sampled (usually the median lobe) so that the tissue on either side of the ligature was pale in colour but was not sheared. The liver sample was then cut off distal to the blood supply, blotted free of excess perfusate, and quickly frozen in liquid nitrogen, (either by freeze-clamping or by dropping the tissue in the liquid). This procedure generally ensured that two samples could be taken from a perfused liver without significant escape of perfusate. The second of a pair of biopsies from a single liver perfusion was made by the same method which was employed if only a single liver sample was to be taken at the end of the perfusion. In this procedure the roller clamp was turned off the moment before the liver sample (left lateral lobe) was excised with scissors, blotted free of excess medium and frozen in liquid nitrogen.

In some experiments, where the intracellular levels of various intermediates were to be measured, it was considered desirable to ensure that no time elapsed between the severance of the blood supply to the tissue and its subsequent fixing in liquid nitrogen. In these experiments, the liver was freeze-clamped in situ using small freeze clamping tongs adapted for single handed operation which had been pre-cooled in liquid nitrogen. When the tissue was freeze-clamped in this way, it was necessary to dissect the frozen liver free of portions of gut etc. which were also likely to be clamped, before any tissue extracts were prepared.

In the experiments described in the present study, significant

metabolic differences were not found to exist between the two major lobes of the liver (left lateral and median), as shown by analysis of simultaneous samples.

2.4.5 The Collection and Assay of $^{14}CO_2$ Released by the Perfused Liver.

In some of the experiments involving the perfusion of the liver with ^{14}C labelled precursors, it was required to collect the $^{14}CO_2$ in the effluent gas from the gas exchanger and measure its radioactivity. CO_2 was collected by bubbling the effluent gas through two wash bottles containing 250 mls. and 50 mls. respectively of 2M NaOH in the apparatus shown in figure 9. CO_2 collection was confined to the portion of the experiment where radioactive substrates were present in the perfusate and the 30 mins. after the liver was removed from the apparatus, thus ensuring almost complete removal of $^{14}CO_2$ from the perfusate. The system was made gas tight by the use of quickfit fittings to the gas exchanger and collecting vessel and the sealing of the perfusion platform to the collecting vessel with plasticine. The negative pressure from the filter pump was carefully regulated so that the net pressure of the gas phase in the system was just less than atmospheric (as shown by the manometer), and the perfusate was not frothing.

The (^{14}C) -sodium bicarbonate in 1 ml. of each of the NaOH solutions was precipitated by reaction with barium chloride in the cold. The barium carbonate thus formed was collected by fitration and washed with methanol before

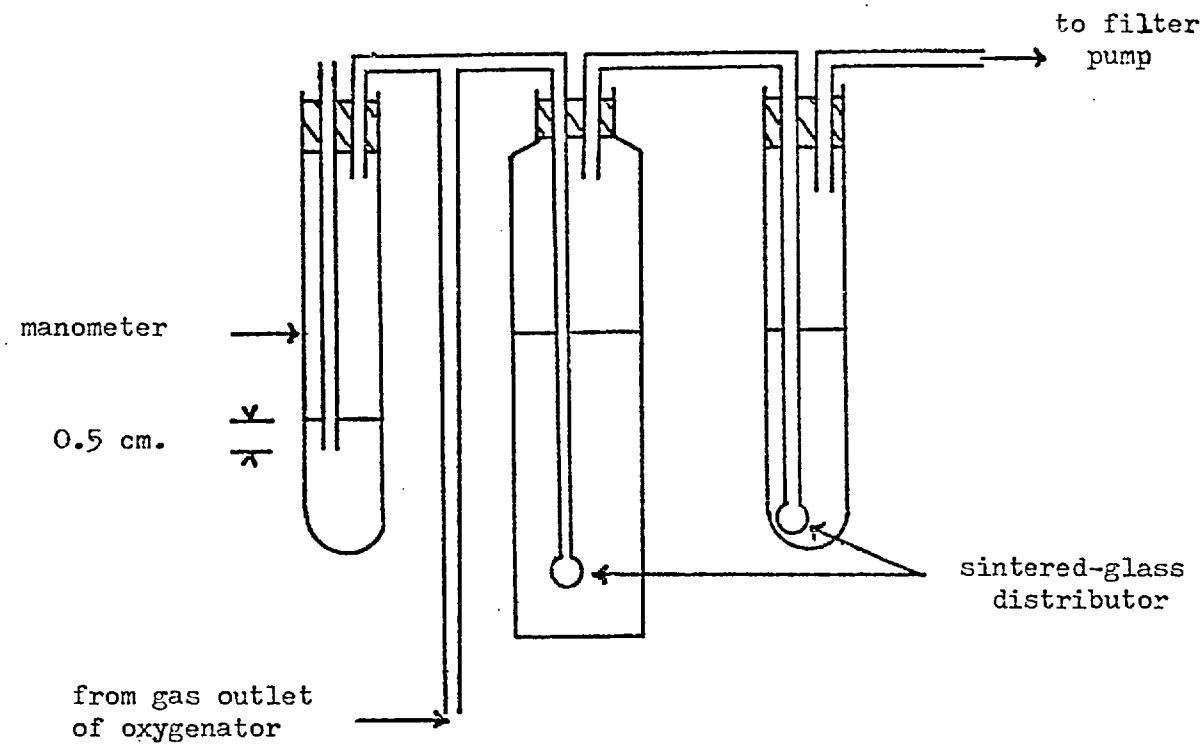


Fig. 9 Apparatus for the collection of CO_2 released by the perfused liver.

The effluent gas from the oxygenator was drawn through the two wash bottles containing 2M NaOH by means of a filter pump fitted to the mains water supply. The pressure in the system was maintained at 0.5 cm. below atmospheric in order to minimise the leakage from the system of $^{14}CO_2$ released by the liver. For further explanation see text.

scintillant was added (1 ml. 2-methoxyethanol + 12 mls. toluene containing 0.8% (w/v) butyl PBD) and its radioactivity measured as described in section 2.7.5.

The recovery of(^{14}C)-carbonate by barium chloride precipitation was tested using labelled sodium bicarbonate of known radioactivity and was found to be in excess of 98%. The efficiency of collection of $^{14}CO_2$ from the gas exchanger by the apparatus shown in figure 9 was similarly tested by liberating with acid a known amount of $^{14}CO_2$ from ^{14}C sodium bicarbonate in circulating perfusion medium. By this method the efficiency of collection of CO_2 was found to exceed 90%.

2.5 Experiments in Vivo.

2.5.1 The Measurement of Fatty Acid Synthesis.

The total rate of de novo fatty acid synthesis was measured in vivo using $^{3}H_2O$. Conscious male rats weighing 180 gms. were injected with $^{3}H_2O$ (5 m Ci in 0.1 ml.) at zero time. After a period of one hour the animals were killed by cervical fracture and samples of liver and epididymal adipose tissue were rapidly frozen in liquid nitrogen. The radioactive fatty acid content of these tissues was then analysed as described in subsequent sections. A sample of blood was taken from the dorsal aorta at the time of death in order to determine the specific radioactivity of the body water.

In these experiments (^{14}C)-glucose was injected at the same

time as $^{3}H_2O$. Since the injected glucose is likely to be rapidly metabolised in these experiments, calculation of total rates of fatty acid synthesis from glucose is not possible. However, the ratio :

$$\frac{^{14}C \text{ d.p.m. } g^{-1}}{^{3}H \text{ d.p.m. } g^{-1}}_{\text{Adipose}} \Big/ \frac{^{14}C \text{ d.p.m. } g^{-1}}{^{3}H \text{ d.p.m. } g^{-1}}_{\text{Liver}}$$

provides a measure of the relative importance of glucose as a precursor of hepatic fatty acids as compared with adipose tissue fatty acids, (Hems et. al. 1975a).

In some of these experiments, as in those subsequently described for the determination of net hepatic glycogen accumulation in vivo, rats were pretreated with an oral dose of glucose. In this procedure, rats were lightly anaesthetized with diethyl ether and 2 ml. of 2 M glucose was introduced into the stomach using a syringe fitted with a no. 4.F.G. 'Portex' plastic canula. The concentration of glucose and lactate in the hepatic portal vein after such pretreatment has been measured in experiments in which single rats were anaesthetized and their hepatic portal blood sampled at various times after glucose administration. The results of these experiments are shown in figure 10.

2.5.2 The Measurement of Net Glycogen Accumulation.

In these experiments, 48 hr. starved rats were anaesthetized

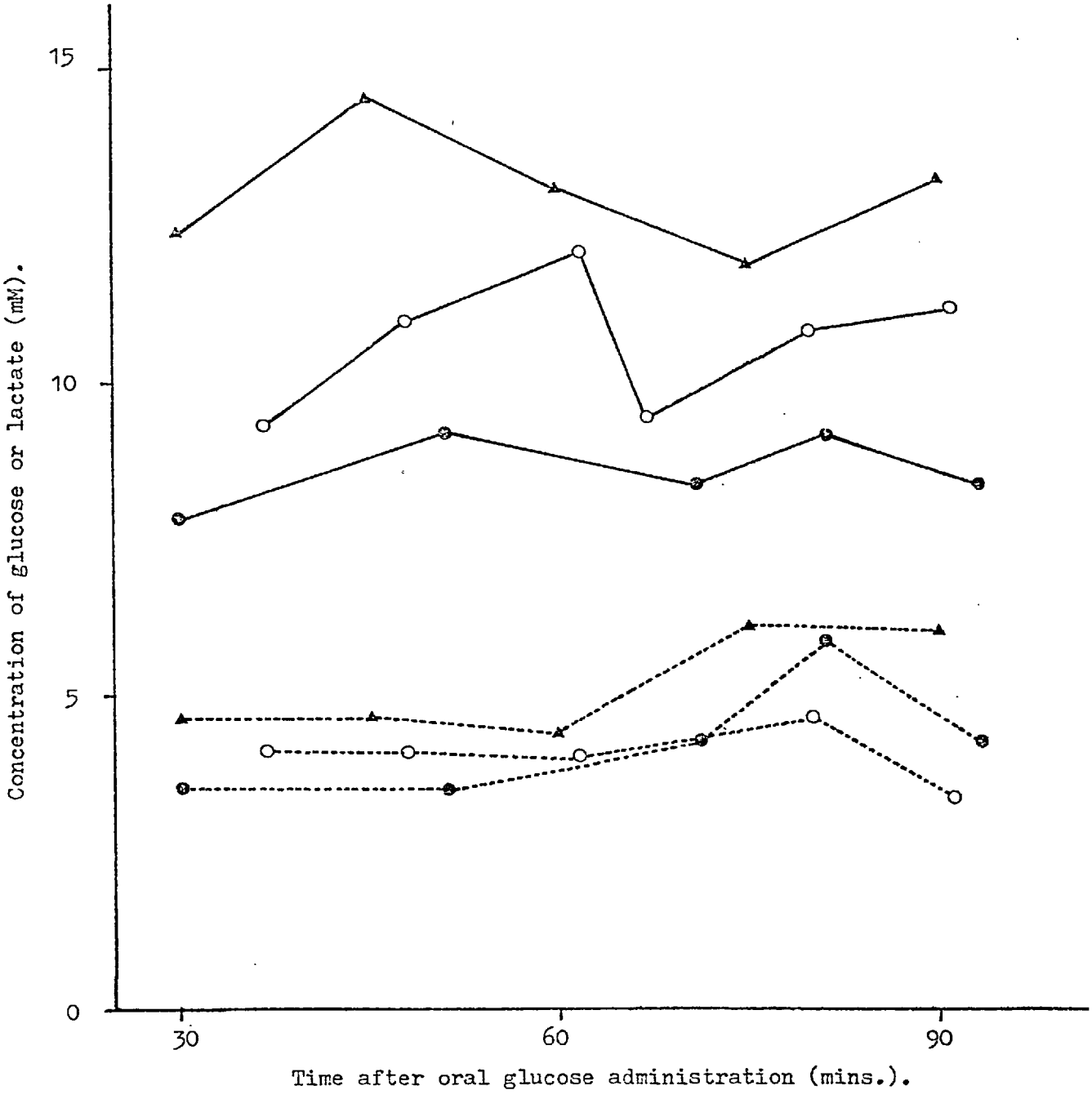


Fig. 10 The effect of oral glucose upon glucose and lactate concentration in the hepatic portal vein.

2 mls. of 2M glucose was administered to lightly anaesthetised normal (▲), adrenalectomized (●) or sham-operated (O) rats at time zero by means of a stomach tube. Single rats were anaesthetised at the times indicated and a blood sample was taken from the hepatic portal vein in a heparinised syringe. Plasma glucose (solid lines), and lactate in a PCA extract of whole blood (dotted lines) were assayed as described in the text.

with Nembutal (0.1 ml./100 gms.) and a tail vein was cannulated with a 1 ml. syringe and 25 G needle containing 0.9% (w/v) sodium chloride. When the needle had been inserted in a tail vein, 0.1 ml. of 0.9% sodium chloride was injected into the vein and the syringe was disconnected, leaving the needle in place. If the vein had been successfully cannulated,a small backflow of blood into the body of the needle was observed. If no backflow occurred, then the needle was removed and the cannulation procedure repeated. A male Luer adaptor was then used to connect the needle to the infusion tube from which substrates (generally glucose) could be pumped into the tail vein with the aid of a Delta pump (Watson - Marlow, U.K.).

Five minutes after the infusion had begun a liver sample was taken for the determination of initial glycogen content. A small transverse incision was made in the ventral body wall just to the left of the midline and about 1 cm. posterior to the diaphragm. Through this incision the left lateral lobe of the liver was exposed and ligatured as described for the perfusion experiments. The liver sample was then excised and frozen quickly in liquid nitrogen. The stump of the left lateral lobe was blotted with a saline soaked pad of tissue which was left in contact with the liver as it was returned to the abdominal cavity. This procedure encouraged clot formation and thus minimised haemorrhage.

The animal was then kept warm by the proximity of an electric lamp and the incision in the body wall protected by a saline soaked tissue. Sixty minutes after the first liver biopsy, a second liver sample (median lobe) was taken by the same procedure and a heparinised blood sample withdrawn from the dorsal aorta

before the animal was killed by exsanguination.

2.5.3 The Measurement of Various Hormonal Effects upon Intracellular Adenosine -3', 5' Cyclic Monophosphate Concentration.

Fed rats were anaesthetized with Nembutal (0.15ml./100 gms.) and placed near an electric light bulb to keep them warm. A transverse incision was made in the body wall and the intestine was slightly displaced onto a saline soaked tissue so as to expose the hepatic portal vein. Preliminary experiments indicated that this procedure elevated the hepatic concentration of cyclic AMP. Since these experiments were designed to detect the smallest of hormonally induced increases in hepatic cyclic AMP, it was considered desirable to allow basal cyclic AMP levels to become re-established before any hormone was administered to the animal. Thus, after a period of 20 mins. had elapsed, hormones in 0.25 m NaCl were injected into the hepatic portal vein at the following doses : glucagon 1.0 μgm, adrenalin 1.5×10^{-8} moles, (8-arginine)-vasopressin 10 or 100 m Units; 0.9% NaCl alone was injected as a control. After the injection, the needle and syringe were left in position in the vein to prevent haemorrhage. At various time intervals after hormone injection, liver samples (median lobe) were taken and rapidly frozen in liquid nitrogen. The samples were immediately homogenised with 10 volumes of 6% (w/v) trichloracetic acid in preparation for the measurement of cyclic AMP. A portion of each sample was also kept frozen for the assay of glycogen phosphorylase (assays performed by Dr. P. Whitton).

2.6 Separation Techniques.

2.6.1 The Extraction and Isolation of Lipids from Tissues and Perfusate.

Lipids were extracted from tissues and perfusate either by direct saponification of the material (Shigeta and Shreeve, 1966) followed by fatty acid extraction with light petroleum (Brunengraber et. al. 1973), or by extraction with chloroform methanol (2:1) (Folch et. al. 1957).

In experiments where only a measurement of total fatty acid synthesis was required, then samples of frozen tissue (liver or adipose tissue, 100 - 300 mg.) or cell free perfusate (2 mls.) were added to ethanolic potassium hydroxide in a quickfit tube. The final concentration of potassium hydroxide was 20% and that of ethanol was 50% in a total volume of 5 mls. The tubes were greased (Silicone high vacuum grease, Edwards, U.S.A.), stoppered and heated in a water bath at 90°C for 3 hrs. to ensure complete saponification of the lipids.

The tubes were then allowed to cool and non-saponifiable lipids were extracted twice by shaking with light petroleum (40 - 60°). The light petroleum extract was evaporated to dryness at 70°C under a stream of nitrogen and the free cholesterol was precipitated with digitonin as described by Brunengraber et. al. (1973). The residue of non-saponifiable lipids was first taken up in 5 mls. of acetone/ethanol (1:1 v/v), acidified with one drop of 10% acetic acid and precipitated with 2 mls. of 0.5% digitonin in 50% ethanol overnight. After spinning at 2500 g. for 5 minutes, the supernatant was removed by aspiration and the pellet washed

with 6 mls. of acetone/ether (1:1 v/v). The precipitate was spun down once more, washed with 6 mls. of ether and finally resuspended in 1 ml of 2 methoxyethanol prior to the determination of its radioactivity by liquid scintillation spectrometry.

After the extraction of non-saponifiable material and the precipitation of cholesterol, one drop of phenolphthalein was added to each saponified sample which was then acidified with 11 M HCl. The free fatty acids thus released from solution were extracted by shaking three times with petroleum ether (40 - 60°).

The petroleum ether fatty acid extracts were then transferred to a scintillation vial and dried under a stream of nitrogen (thus minimizing fatty acid oxidation) prior to the estimation of radioactivity by liquid scintillation spectrometry (Section 2.7.5). Using tubes containing the normal saponification mixture (q.v.) and known amounts of radioactive fatty acids, it was shown that the extraction procedure described above removed 99% of the added radioactivity in three washes.

In preparation for the analysis of the distribution of newly formed fatty acids among the various lipid classes, samples of liver or cell free perfusate were homogenised with 20 vols. of chloroform:methanol (2:1 v/v) in a vortex blender (Folch et. al. 1957). The samples were then left at room temperature overnight and filtered under vacuum. 0.2 vols. of 0.1 M potassium chloride was added to each filtrate and the tube shaken vigorously. The two phases were allowed to separate and the aqueous phase was removed by aspiration. The infranatant was then washed by shaking with a synthetic upper phase consisting of chloroform:methanol: 0.1 M KCl:H_2O (3:48:47:1 by vol). When the separated upper phase

had been removed by aspiration, the chloroform phase was evaporated to dryness at 80°C under a stream of nitrogen. The lipid extracts were then stored in chloroform at -20°C for up to 3 days in order to minimise autoxidation of polyenoic fatty acid prior to t.l.c. procedures.

The extraction of duplicate liver samples containing radioactive fatty acids by methods including the two described above (direct saponification and chloroform methanol extraction) yielded the results shown in table 1. It is clear that both methods of extraction removed similar amounts of radioactive fatty acids from the samples, but about 10 % of the total radioactivity was lost during the washing procedure applied to the chloroform methanol extracts. This radioactivity was presumably in very polar phospholipids or short chain fatty acids.

2.6.2 The Separation of Lipid Classes by Thin Layer Chromatography.

Washed chloroform methanol extracts were separated into lipid classes by a technique based upon the double solvent development technique of Freeman and West (1966) using 0.25 mm. thin layers of silica gel G (E. Merck, Darmstadt, West Germany). The plates were developed sequentially in diethyl ether/toluene/ethanol/acetic acid (50:40:2:0.2 by vol.) to within 5 cms. of the top of the plate, followed by n-hexane/diethyl ether (94:6 v/v) which separated cholesteryl esters from triglycerides at the first solvent front. After development, the plates were sprayed with 0.1% methanolic rhodamine 6G and the separated lipids were identified

Table 1 An evaluation of the lipid extraction techniques used in the present study.

Table 1 An evaluation of the lipid extraction techniques used in the present study.

Various procedures used for the extraction of radioactive lipids from tissue were evaluated by comparing the recovery of radioactivity from such procedures with that obtained by directly saponifying the tissue and extracting the acidified soaps three times with light petroleum (40 - 60°) (Shigeta and Shreeve, 1966). The lipid extracts resulting from each of the methods in the table were evaporated to dryness before saponification and extraction as described in the text. Thin layer chromatography (Freeman and West, 1966) of the final light petroleum extract of the saponified lipids indicated that more than 99.2% of the radioactivity on the t.l.c. plate was located in the free fatty acid band. Results, expressed as % radioactivity (d.p.m. g^{-1}) recovered by each procedure as compared with direct saponification, are the mean of two separate determinations using livers from an adrenalectomized and sham-operated rat.

	Extraction procedure	Reference	Recovery of radioactive fatty acids (% of those recovered following direct saponification)	
			^{3}H-labelled	^{14}C-labelled
1)	Direct saponification of tissue	Shigeta and Shreeve (1966)	100.0	100.0
2)	Acetone : ethanol (1:1) extraction		99.8	101.5
3)	Chloroform : methanol (2:1) extraction alone	Folch et. al. (1951)	98.4	104.6
4)	3 + 'Folch wash'	Folch et. al. (1957)	88.6	94.0
5)	3 + 4 + Thin layer chromatography and elution	Freeman and West (1966) and text.	85.8	87.8

under u.v. light at 254 nm. Bands of silica containing various lipid classes were scraped from the plates. Lipids with R_f values greater than those of free fatty acids were eluted with diethyl ether/light petroleum (40 - 60°)/formic acid (50:50:1 by vol.). phospholipids were eluted with chloroform/methanol/ammonia (50:50:1 by vol.) in a modification of the method of Bickerstaffe and Annison (1970). The eluates were evaporated to dryness at 70°C under a stream of nitrogen and their radioactivity measured by liquid scintillation spectrometry.

In an extension to the previously described experiment designed to test the recovery of radioactivity from directly saponified and chloroform/methanol extracted samples, a duplicate liver sample was extracted with chloroform/methanol and washed according to the method of Folch et. al. (1957) as previously described. This sample was then separated into lipid classes by thin layer chromatography and the radioactivity in the eluates from the whole plate measured by liquid scintillation spectrometry. From the results presented in table 1 it is clear that the loss of radioactive fatty acids incurred during the tlc procedure described above was about 4%.

2.6.3 The Separation of Fatty Acids According to their Degree of Unsaturation by Thin Layer Chromatography.

Fatty acids extracted from directly saponified samples by the method of Brunengraber et. al. (1973) were methylated in 14% (w/v) methanolic BF_3 for 5 mins. at 100°C (Metcalf and Schmitz, 1961)

and extracted three times with light petroleum (40 - 60°). An aliquot of the fatty acid methyl esters (FAME) thus prepared (60 mg. approx.) was applied in a band to a t.l.c. plate. The plates were spread with a 0.5 mm. layer of silica gel G prepared in 10% silver nitrate, and activated at 110°C for 90 mins. The plates were developed in light petroleum/diethyl ether (90:10 v/v), allowed to dry, and sprayed with 0.1% methanolic rhodamine 6G. The bands were identified by brief visualisation under u.v. at 254 nm., scraped into vials and eluted with diethyl ether. The diethyl ether extracts of FAME were evaporated to dryness at 70°C under a stream of nitrogen and their radioactivities determined by liquid scintillation spectrometry.

2.7 Analytical Methods.

2.7.1 The Assay of Free Fatty Acids in Saponified Perfusion Medium.

The concentration of circulating free fatty acids in perfusate was determined by a modification of the method of Lauwerys (1969). 2 mls. of cell free perfusate was shaken with 20 mls. of isopropanol/heptane/$2NH_2SO_4$ (40:10:1 by vol.) in a glass stoppered test tube. 12 mls. of heptane and 8 mls. of water were then added and the tube shaken again. The upper (heptane) phase was transferred to a screw top vial, the contents of the tube washed again with a similar volume of heptane and the washings merged. The heptane extract was then evaporated to dryness at 70°C under a stream of nitrogen and the extracted fatty acids were taken up

in a known volume of heptane.

A 3 ml. aliquot of the heptane solution of free fatty acids was transferred to another tube containing 3 mls. of chloroform and 3 mls. of a copper reagent containing :

7 ml. triethanolamine

0.3 ml. glacial acetic acid

3.25 gm. $Cu(NO_3)_2 3H_2O$

6.25 gm. $K_2 SO_4$

17.0 gm. $Na_2 SO_4$

in a final volume of 100 mls.

This tube was then shaken for 2 mins. with a mechanical shaker, during which time the free fatty acids were converted to chloroform soluble copper soaps. Having been thus shaken, the tube was centrifuged for 10 mins. at 2500 g. to ensure that no copper reagent was left adhering to the side of the tube in the upper phase. 3 ml. of the upper chloroform heptane phase was then mixed with 0.5 ml. 0.1% (w/v) sodium diethyldithiocarbamate dissolved in n-butanol in a glass cuvette (1 cm. light path). A yellow colour developed immediately as the copper reacted with the diethyldithiocarbamate, and the absorbance was measured at 440 nm. against a reagent blank. Standards of palmitic acid bound to de-fatted bovine serum albumin were prepared as described in section 2.3.2 so that the final albumin concentration was similar to that of the cell free perfusates. These standard solutions were then extracted and assayed as described above so that a standard curve for the assay could be constructed over the range of 0.2 - 2.0 mM albumin bound palmitic acid. Comparison of the standards prepared as described

above, with standards prepared by dissolving palmitic acid directly in chloroform/heptane (1:1 v/v) immediately prior to the assay procedure, indicated that the recovery of albumin bound palmitic acid was greater than 96%. Hence, for most FFA assays, the palmitic acid standards were dissolved directly in chloroform/heptane 1:1(v/v).

2.7.2 The Estimation of Circulating Glucose and Hepatic Glycogen.

The Hydrolysis of Hepatic Glycogen.

A sample of frozen liver (0.1 - 0.5 gm.) was ground to a powder in a mortar which had been pre-cooled in liquid nitrogen, and weighed into a boiling tube. 10 volumes of 30% KOH (w/v) were added and the contents of the tube heated to 100°C in order to ensure that the KOH had thoroughly permeated the tissue. Samples could be stored in this state for several days if required. The samples were then heated in a boiling water bath for 30 mins. and the glycogen was precipitated with 3 vols. of absolute ethanol at 4°C overnight. The glycogen thus precipitated, was then sedimented by centrifugation at 10,000 r.p.m. for 20 mins. The supernatant was carefully poured off and the pellet was resuspended in 20 mls. of distilled water with the aid of a motor driven pestle. An aliquot of each glycogen suspension containing 0.05 - 0.3 moles of glycogen - glucose was then hydrolysed to glucose by incubating at 37°C for 1 hr. in 0.025 M sodium acetate buffer (ph 4.8) containing 7 units of amylo-α-1,4-α-1,6-glucosidase (EC 3.2.1.3)in a final volume of 1 ml.

The Estimation of Glucose.

Glucose in serum, cell free perfusate, perchloric acid extracts or glycogen hydrolysates was determined by a glucose oxidase method (Krebs et. al. 1964). Samples were prepared from the above mentioned sources, containing 0.05 - 0.3 μmoles of glucose in 1 ml. of water. To each sample was added 2.5 ml. of a solution containing 250 I.U. glucose oxidase, 150 I.U. peroxidase and 0.5 ml. 1% ortho-dianisidine in 95% ethanol made up to a final volume of 100 mls. with 0.5 M sodium phosphate buffer (ph 7.3) containing 0.1 M tris. The samples were incubated with the enzyme solution for 1 hr. at 37°C, and the absorbance of the oxidised dianisidine was measured at 440 nm. against a reagent blank. Standard samples of glucose dissolved in water were prepared and the standard curve for the assay was found to be linear up to 0.3 μmoles glucose/cuvette.

2.7.3 The Determination of Glycolytic and other Intermediates Involved in Carbohydrate Metabolism.

Metabolic intermediates were determined enzymatically in neutralised perchloric acid extracts of liver by coupling the enzymatic conversion of a particular metabolite to a specific dehydrogenase reaction in which pyridine nucleotides are produced or consumed. An example of this principle is the assay for citrate described by Moellering and Gruber (1966) using the enzymes citrate lyase and malate dehydrogenase. In this assay,

citrate lyase first catalyses the conversion of citrate to oxalacetate and acetyl CoA:

$$\text{citrate} + \text{ATP} \xrightarrow[\text{H-S-CoA}]{} \text{oxalacetate} + \text{acetyl CoA} + \text{ADP} + \text{Pi}$$

In the presence of malate dehydrogenase, oxalacetate is then hydrogenated to produce malate with the associated oxidation of NADH to NAD^+

$$\text{oxalacetate} + \text{NADH} + H^+ \rightleftharpoons \text{malate} + NAD^+$$

A number of metabolic intermediates were determined by means of assays of this type; a full list is to be found in table 2. Frequently, several metabolites were determined in a single cuvette by the separate addition of different enzymes. An example of such an assay is that in which pyruvate, 3 phosphoglycerate, 2 phosphoglycerate and phosphoenolpyruvate were measured in a single cuvette by the sequential addition of lactate dehydrogenase, phosphoglyceromutase, enolase and pyruvate kinase (Czok and Eckert, 1963).

$$\text{pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{lactate} + NAD^+$$

$$\text{phosphoenolpyruvate} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP}$$

$$\text{2 - phosphoglycerate} \rightleftharpoons \text{phosphoenolpyruvate}$$

$$\text{3 - phosphoglycerate} \rightleftharpoons \text{2 - phosphoglycerate}$$

Assays of this type were easily performed in individual spectrophotometer cuvettes. Each cuvette contained a sample of the neutralised acid liver extract and any other reactants (eg. pyridine nucleotides) in a suitable buffer. The

Table 2 A summary of the procedures employed for the assay of metabolic intermediates in the present study.

Metabolite	Enzyme	Reference
Pyruvate	Lactate dehydrogenase	Czok and Eckert (1963)
Phosphoenol-pyruvate	Pyruvate kinase	
2-Phosphoglycerate	Enolase	
3-Phosphoglycerate	Phosphoglyceromutase	
Pyruvate	Lactate dehydrogenase	Adam (1963)
ADP	Pyruvate kinase	
AMP	Myokinase	
Lactate	Lactate dehydrogenase	Hohorst (1963a)
Malate	Malate dehydrogenase	
Glycerol-3-phosphate	Glycerol-3-phosphate dehydrogenase	
Dihydroxyacetone phosphate and glyceraldehyde-3-phosphate	Triose phosphate isomerase and glycerol -3-phosphate dehydrogenase	Bucher and Hohorst (1963)
Fructose-1, 6-diphosphate	Aldolase	
Glucose-6-phosphate	Glucose-6-phosphate dehydrogenase	Hohorst (1963b)
Fructose-6-phosphate	Phosphohexose isomerase	
Glucose-1-phosphate	Phosphoglucomutase	
ATP	Hexokinase	
β Hydroxybutyrate	β-Hydroxybutyrate dehydrogenase	Williamson and Mellanby (1963)
Citrate	Citrate lyase Malate dehydrogenase	Moellering and Gruber (1966)
Aspartate	Glutamate-oxalacetate transaminase Malate dehydrogenase	Pfleiderer (1963)

optical density of the reaction mixture was then allowed to stabilise and was noted prior to the addition of the enzymes. Upon addition of these enzymes the optical density at 340 nm. was observed to change as pyridine nucleotides were interconverted. Enzymes were also added to a blank cuvette containing the full reaction mixture together with a neutralised perchloric acid blank in place of the liver extract. Calculations of the amount of pyridine nucleotide consumed were based upon the difference between the change in optical density in the experimental cuvettes and the change in optical density in the reagent blank cuvette.

Since NAD(P)H has an extinction coefficient of 6.22 μmol^{-1}. cm^{-1} at 25° C, these assays were performed in a total volume of 3.11 mls. so that the net extinction change was equal to twice the change in concentration of NAD(P)H in the assay mixture. Assays of this type were frequently tested for total recovery of substrate by adding a known amount of substrate to 3.II ml of reaction mixture.

2.7.4 The Assay of Cyclic Nucleotides in Liver and Perfusate.

The preparation of tissue extracts.

Samples of liver were freeze-clamped in liquid nitrogen-cooled tongs and pulverised in a pre-cooled pestle and mortar. 0.1 - 0.2 g. of the frozen powder was extracted with 10 vols. (w/v) of 6% (w/v) trichloracetic acid (TCA) by homogenisation with a vortex blender. 0.2 - 0.4 ml. of the extract thus obtained was washed four times with water-saturated diethyl ether in order to remove the TCA. The washed extract was dried over P_2O_5 in a vacuum dessicator and dissolved in 0.4 ml. of 0.05M Tris-HCl assay buffer (pH 7.4) containing 8 mM theophylline and 2 mM 2-mercaptoethanol in preparation for the assay procedure.

Extracts of perfusion medium were prepared in one of the following two ways. In preparation for the assay of cyclic AMP, perfusion medium was spun to remove erythrocytes and mixed with an equal volume of the assay buffer described above. The samples were then heated for 3 mins. at 100°C in a water-bath and centrifuged at 5000 r.p.m. for 5 mins. to remove the protein. Perfusate samples intended for cyclic GMP determination were extracted with an equal volume of 10% w/v TCA, washed with water-saturated diethyl ether, dried and taken up in the assay buffer as described for the liver extracts.

The assay of cyclic AMP.

Cyclic AMP in tissue extracts was measured by a saturation assay technique based upon that described by Brown et. al. (1971). The assay utilises a specific binding protein for cyclic AMP extracted from bovine adrenal glands. For the present study, the binding protein was kindly prepared by Dr. I. Das (Dept. of Biochemistry, Imperial College) as described by Brown et. al. (1971).

Assay tubes were prepared containing 0.05 ml. of either a tissue extract or a known amount (0 - 15 pmol) of cyclic AMP, 0.05 ml. tritiated cyclic AMP (9.1 mCi/mg., approx. 8 n Ci), 0.1 ml. of the binding protein diluted with an appropriate amount of assay buffer, and assay buffer to a final volume of 0.35 ml. Cyclic AMP standards were dissolved in the appropriate solvent for each assay. Thus, for the assay of hepatic cyclic AMP, standard samples were dissolved in 5.45% (w/v) TCA which had been washed four times with water saturated diethyl ether. For the assay of cyclic AMP in cell free perfusate, standards were prepared in half strength assay buffer containing 1% bovine serum albumin which had been heated for 3 mins. at 100°C in a water bath. After mixing, an aliquot (0.05 ml.) was removed from each tube and dissolved in 2 ml. of 2-methoxyethanol in a scintillation vial. 12ml. of scintillant consisting of 0.8% butyl PBD in toluene was added and the radioactivity determined as described in section 2.7.5. The assay tubes were incubated at 4°C for 90 minutes before the addition to each of 0.1 ml. of a 10% (w/v) suspension of charcoal (Norit GSX, Norit Clydesdale, Glasgow, U.K.)

in assay buffer containing 2% (w/v) bovine serum albumin (Fraction V). After mixing, the tubes were centrifuged at 1200 g. for 15 mins. at 4°C and 0.1 ml. of the supernatant (i.e. the protein bound fraction) was taken up in 2-methoxyethanol in preparation for liquid scintillation counting as described in section 2.7.5. Standard curves were plotted as the percentage of radioactivity bound against the logarithm of the amount of nucleotide in the standard samples (fig. 11). The amount of cyclic AMP in the unknown samples was determined by reference to the appropriate standard curve. Standard curves were found to be linear between 1 - 5 p mol cyclic AMP per tube.

The recovery of cyclic AMP added to tissue extracts was found to vary between 95-115% and this recovery was not altered by the addition of arginine vasopressin to the tissue prior to extraction. Standard curves were constructed with liver and perfusate blanks containing a higher concentration of arginine vasopressin than that which might be found in the tissue extracts. These curves were identical to those constructed in the absence of arginine vasopressin (fig. 11); thus it is clear that the hormone did not affect the binding of cyclic AMP to the assay protein during the assay procedure.

The assay of cyclic GMP.

Tissue extracts prepared as described previously were assayed for cyclic GMP by radioimmunoassay (assays kindly performed by Dr. K. Siddle, University of Cardiff). The radioimmunoassay

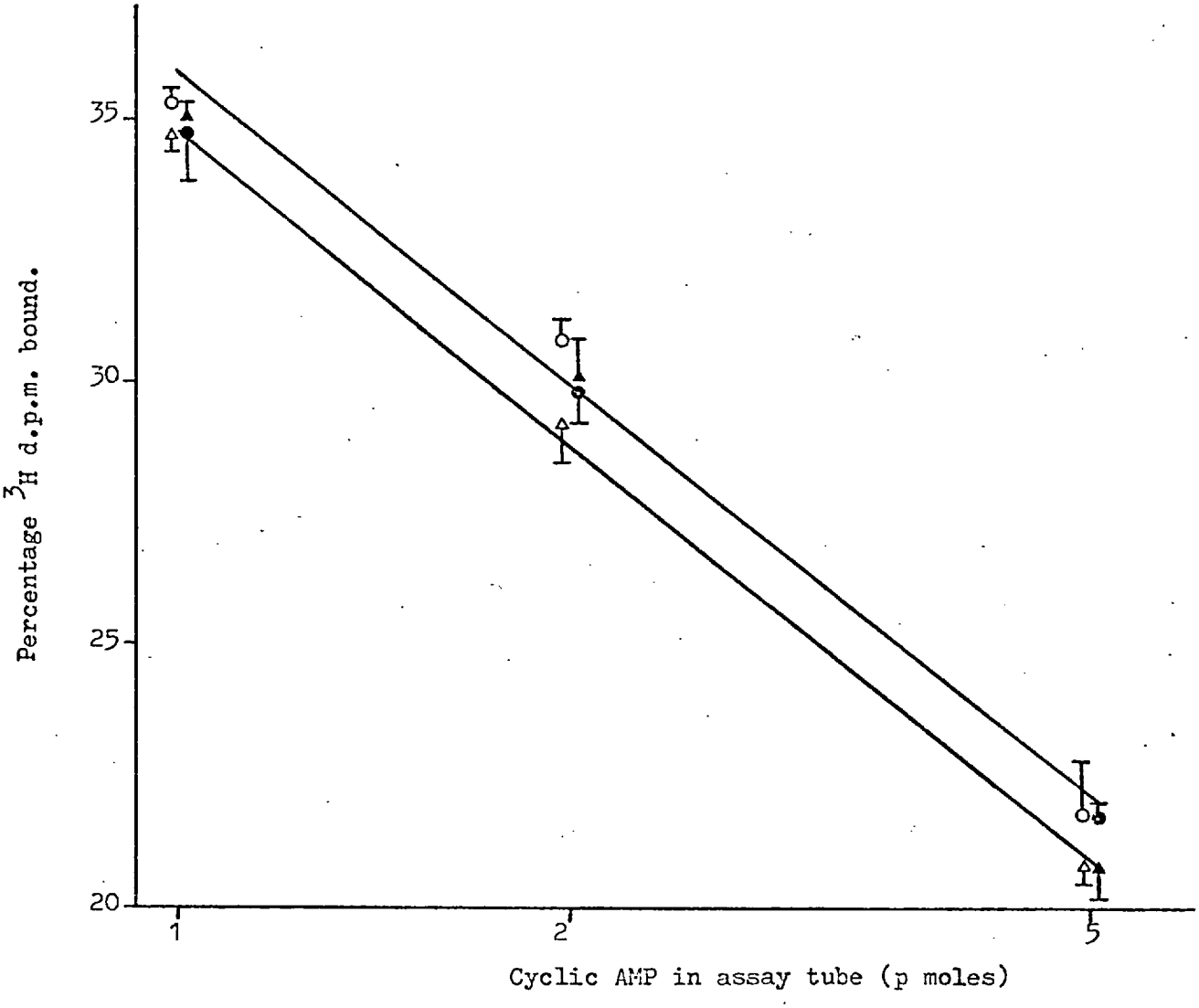


Fig. 11 Standard curve for the assay of cyclic AMP :- The influence of vasopressin upon nucleotide binding.

Cyclic AMP was assayed as described in the text. Standard samples were prepared in ether washed 5.45% TCA (△) for the assay of hepatic cyclic AMP or boiled half strength assay buffer (○) for the assay of perfusate cyclic AMP. The addition of arginine vasopressin to the standard samples did not affect the standard curves for the assay of either hepatic (▲) or perfusate (●) cyclic AMP. Points are means ± S.E.M. of 3 determinations.

utilised antibodies to cyclic GMP (bound as a hapten) and was found to be sensitive to 0.01 p mol of cyclic GMP per assay tube.

2.7.5 Liquid Scintillation Spectrometry.

The preparation of samples for liquid scintillation spectrometry.

Aqueous samples of radioactive materials (e.g. perfusion medium) were taken up in a minimum volume of 2-methoxyethanol (0.5 - 2.0 mls.) before the addition of 12 mls. of a scintillation fluid containing 8 g. butyl PBD/litre toluene.

Radioactive lipid extracts (free fatty acids from direct saponifications or complex lipids eluted from t.l.c. plates) were evaporated to dryness at 70°C under a stream of nitrogen before the addition of 12 mls. of scintillation fluid.

Standard samples variably quenched with chloroform were prepared from ^{14}C and ^{3}H labelled hexadecane in the standard scintillation fluid.

The determination of ^{3}H and ^{14}C radioactivity by liquid scintillation spectrometry.

Aqueous samples of tritiated cyclic AMP from cyclic AMP determinations were prepared as described previously. The radioactivity in each sample was measured in a Packard Tri-Carb liquid scintillation spectrometer. The ^{3}H d.p.m. were calculated from

the recorded c.p.m. by means of a channels ratio quench correction technique using chloroform quenched standard samples (Baillie, 1960). This quench correction was checked from time to time with ^{3}H hexadecane as an internal standard. The efficiency of ^{3}H counting was about 20 - 30%.

In samples doubly labelled with ^{14}C and ^{3}H the two isotopes were assayed simultaneously as described by Hendler (1964). The three channels of a Packard Tri-Carb liquid scintillation spectrometer were adjusted so that they covered the energy spectrum of an unquenched doubly labelled sample as shown in figure 12. The principal criteria for choosing the particular discriminator settings were as follows :

1. The lower energy limit of channel C (S_1) was adjusted just above zero so that background electrical noise was not detected. The upper energy limit of channel A (S_5) was adjusted so as to exclude no emission from minimally quenched ^{14}C samples.

2. Minimally quenched tritium was counted in channel A, and S_3 was adjusted so that 99.9% of the radioactivity was excluded from that channel.

3. Using minimally quenched samples of ^{3}H and ^{14}C, S_2 was adjusted so that the sum of the fractional loss of maximum C^{14} radioactivity from channel C plus the fractional loss of maximum ^{3}H radioactivity from the same channel was equal to one.

4. S_4 was adjusted so that a minimally quenched sample of

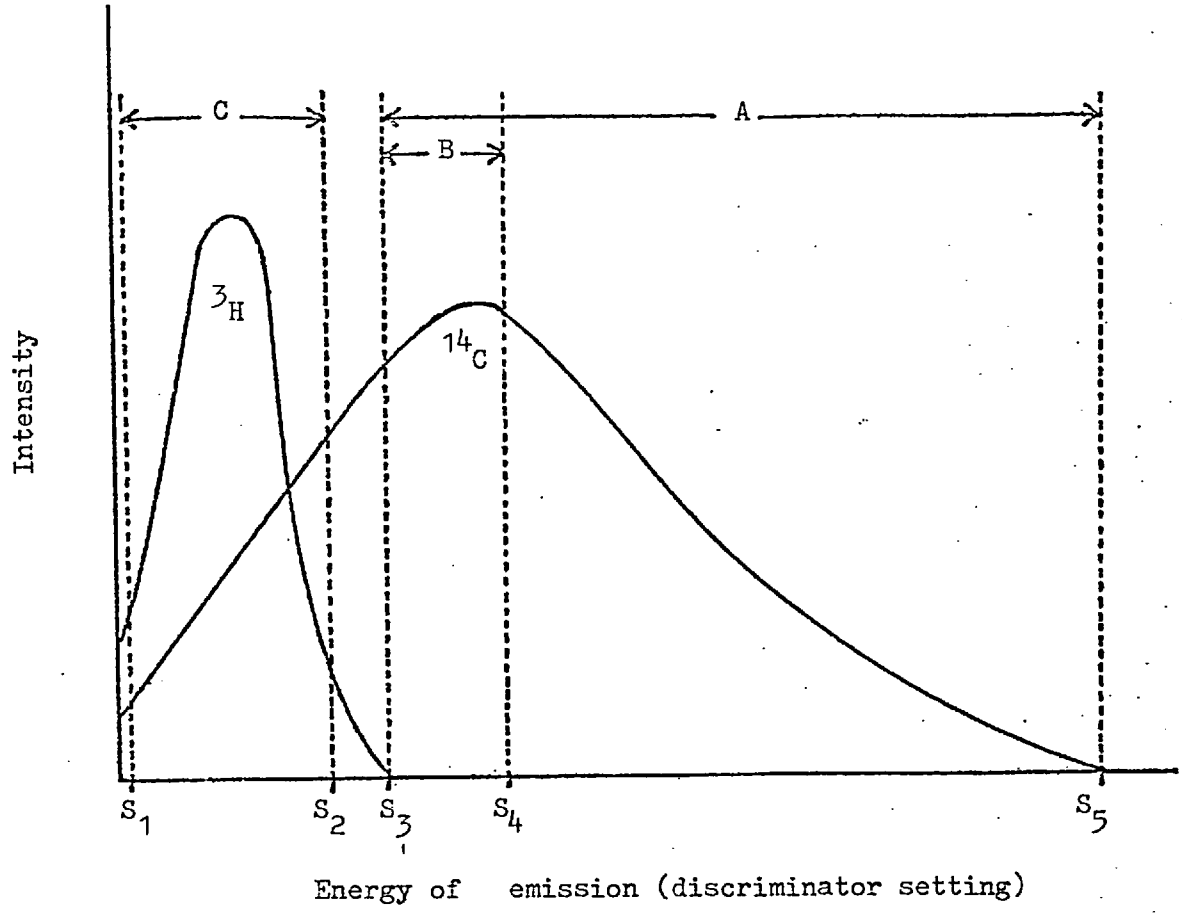


Fig. 12 Discriminator settings relative to the energy spectra of ^{3}H and ^{14}C.

The figure shows the energy spectra of an unquenched doubly labelled sample containing ^{3}H and ^{14}C. The discriminator settings S_{1-5} define the limits of channels A, B and C as shown above, and further explained in the text.

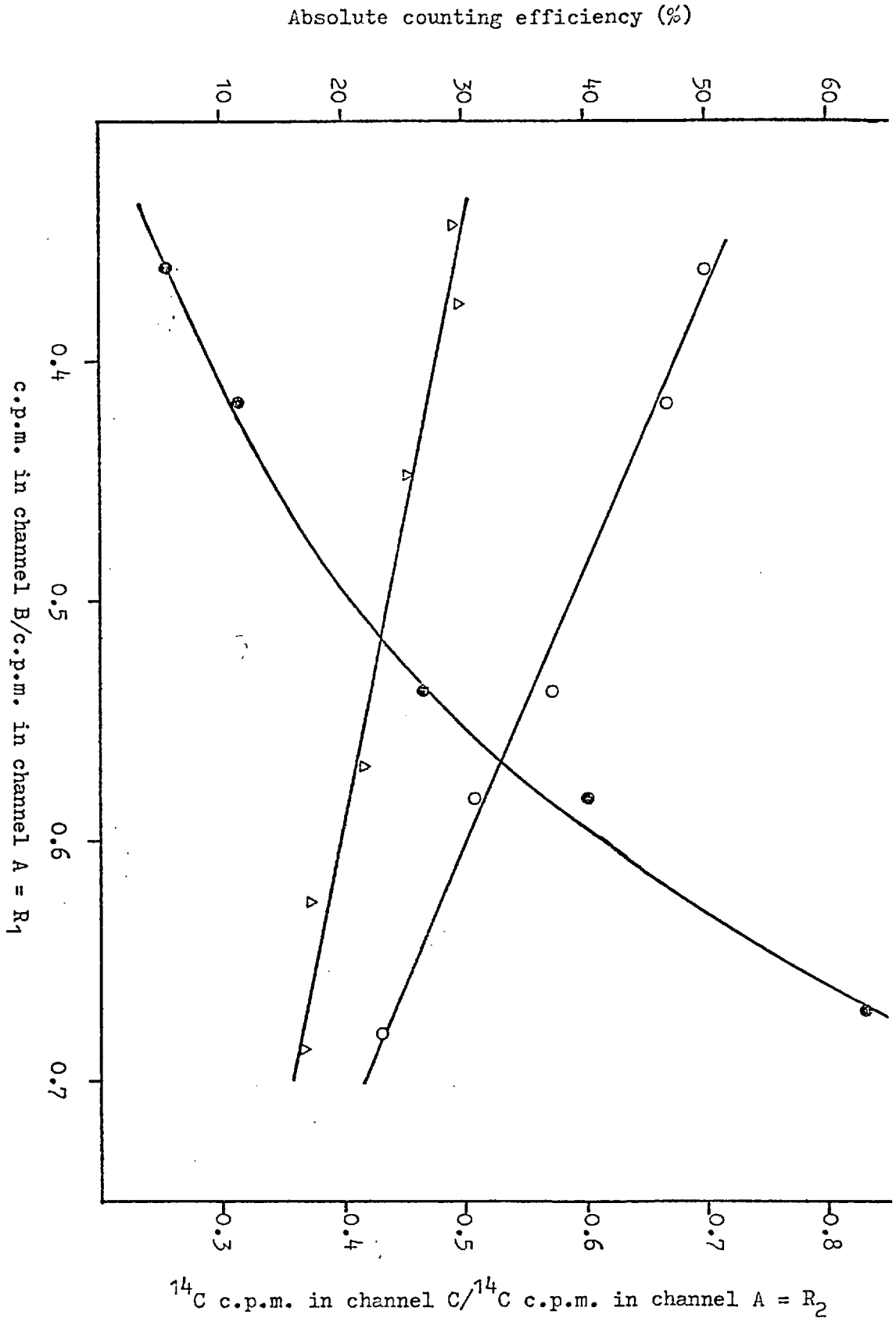
^{14}C produced a ratio of radioactivity detected in channel B to that detected in channel A (ratio R_1) of 0.3.

The radioactivity of unknown doubly labelled samples was calculated by a computerised technique based upon the following method. A series of standard samples were prepared, all of which contained equal amounts of 1-(^{14}C)-hexadecane, half the standards also contained equal amounts of 1-(^{3}H)-hexadecane. The standards were quenched to varying degrees with chloroform so that both those with and those without 1-(^{3}H)-hexadecane covered the same range of quenching (amount of chloroform added 0 - 0.75 ml. per vial). The standard samples were counted in the liquid scintillation spectrometer together with each group of experimental samples. From the counts obtained in channels A and B, a graph was plotted of R_1 against the counting efficiency (i.e. c.p.m. in channel A / ^{14}C d.p.m. in each standard calculated from the data supplied by the Radiochemical Centre, Amersham, Bucks. U.K.) (fig. 13). Using the counts from the standard samples containing 1-(^{14}C)-hexadecane alone, a second graph was constructed of the proportion of the ^{14}C radioactivity detected in channel C to that detected in channel A (ratio R_2) against R_1 (fig. 13). Using this graph, the counts in channel C arising from emission of 1-(^{3}H)-hexadecane in the double labelled standards were calculated by subtracting the counts due to ^{14}C disintegration. Thus a third graph of ^{3}H counting efficiency against R_1 was drawn (fig.13). By reference to these three graphs the ^{14}C and ^{3}H radioactivities (in d.p.m.) of experimental samples were calculated as follows :

Fig. 13 Calibration curves for double isotope liquid scintillation spectrometry.

Fig. 13 Calibration curves for double isotope liquid scintillation spectrometry.

The curves were plotted as described in the text. ● denotes the relationship between R_1 and R_2 determined with standard samples containing 1-(^{14}C)-hexadecane alone of known radioactivity, and quenched with increasing quantities of chloroform. o denotes the relationship between R_1 and the absolute counting efficiency of ^{14}C, also determined with quenched samples of 1-(^{14}C)-hexadecane. Δ denotes the relationship between R_1 and the absolute counting efficiency of ^{3}H determined with standard samples containing both 1-(^{3}H)-hexadecane and 1-(^{14}C)-hexadecane and quenched with increasing quantities of chloroform.


Absolute counting efficiency (%)
10
20
30
40
50
60
0.4
0.5
0.6
0.7
c.p.m. in channel B/c.p.m. in channel A = R_1
0.3
0.4
0.5
0.6
0.7
0.8
^{14}C c.p.m. in channel C/^{14}C c.p.m. in channel A = R_2

^{14}C d.p.m. = (c.p.m. in window A) x 100 / ^{14}C counting efficiency.

^{3}H d.p.m. = c.p.m. in window C - (c.p.m. in window A x R_2) / ^{3}H counting efficiency.

Thus the ^{3}H and ^{14}C radioactivities were calculated on the basis of the channels ratio R_1. This calculation gives satisfactory results as long as the ^{14}C c.p.m. in channels A and B are large enough to be statistically significant and also sufficiently large in relation to the ^{3}H c.p.m. so that the 0.1% overspill of these counts into channels A and B does not significantly alter R_1. It was also important to ensure that the ^{14}C d.p.m. was never greater than that of ^{3}H, or the error in calculating the ^{14}C c.p.m. in channel C became very large and thus produced corresponding inaccuracies in the ^{3}H quench correction. With doubly labelled samples, the efficiency of counting of ^{14}C and ^{3}H were about 45% and 25% respectively. The computerised quench correction used in the present study was validated for various radioactive samples by the use of ^{14}C and ^{3}H labelled hexadecane as internal standards.

2.8 The Calculation of Rates of de novo Synthesis of Fatty Acids and Cholesterol from the Incorporation of ^{3}H from $^{3}H_2O$ and ^{14}C from ^{14}C - Precursors.

The incorporation of isotopes of hydrogen derived from isotopically labelled water provides a valuable technique for the measurement of fatty acid and cholesterol synthesis. Schoenheimer

and Rittenberg (1937) first used 'heavy water' (D_2O) to measure fatty acid synthesis in mice. These same workers previously showed that the C-H bonds in fatty acids are very stable to H atom exchange with water, thus the incorporation of hydrogen (or deuterium) into fatty acids only occurs during synthesis (Schoenheimer and Rittenberg, 1935).

Windmueller and Spaeth (1966) used tritium oxide (tritiated water, ^3H_2O) to measure the synthesis of fatty acids and cholesterol in the perfused rat liver and were the first to calculate molar rates of FA synthesis with this method. These workers expressed their results in terms of the 'relative total activity' (RTA), defined as follows :

$$RTA = \frac{\text{d.p.m. } ^3H \text{ in hepatic lipid / g wet liver}}{\text{d.p.m. } ^3H \text{ / } \mu g \text{ atom H in perfusate } H_2O}$$

Thus, if it is assumed that ^3H_2O is only a small proportion of perfusate water (in practice this was always so), and that neither ^3H nor H are preferentially incorporated into hepatic lipids during synthesis, then RTA = μg atoms of hydrogen from water incorporated, per g. wet liver.

It is known, however, that discrimination against ^2H and ^3H relative to H does occur during hepatic lipogenesis. Rittenberg and Schoenheimer (1937) have shown that if mice are fed on a fat free diet and maintained at a steady deuterium content for up to 96 days by allowing them to drink ^2H_2O, then ^2H concentration in saturated body FA (calculated per total H atoms) equilibrated at 0.44 of that in body water. In similar experiments, Bernhard and Bullet (1943) found that the deuterium content of carcass saturated FA (per total H atoms) was 0.42 of that in body water.

Jungas (1968) incubated rat adipose tissue with $^{2}H_2O$ and ^{14}C glucose and estimated that the apparent incorporation of H into fatty acids was 0.53 of the actual value if $^{2}H_2O$ was used to follow H incorporation. Hence, in the present study a figure of 0.5 has been assumed for the theoretical relative incorporation of ^{2}H into FA during synthesis as compared with H incorporation. The question then arises of the isotope effect for ^{3}H incorporation into FA relative to that of ^{2}H. Experiments in which rats were maintained for 2 weeks with 2% deuterium in body water and a constant $^{2}H_2O$ / $^{3}H_2O$ ratio have indicated a preferential incorporation of deuterium over tritium into FA by a factor of 1.19 (Eidinoff et. al., 1953). Jungas (1968) reported a similar discrimination factor in experiments with incubated rat adipose tissue. Thus, in the present study it was assumed that H from H_2O was preferentially incorporated into FA relative to ^{3}H from $^{3}H_2O$ by a factor of (1.19 / 0.5) = 2.38. Since, in the synthesis of FA, each C_2 unit derived from acetyl CoA has 4 H atoms, the division of RTA by (4 / 2.38) = 1.68 yields the total rate of fatty acid synthesis expressed as incorporation of C_2 units.

Cholesterol synthesis from $^{3}H_2O$ may be expressed in a similar manner. Since cholesterol has 45 C-H bonds and 27 C atoms, the average number of carbon bound hydrogen atoms per C_2 unit in cholesterol is (45 / 13.5) = 3.33. Thus, assuming the same discrimination against ^{3}H as calculated for fatty acid synthesis, the division of the RTA for ^{3}H incorporation into cholesterol by (3.33 / 2.38) = 1.4 yields the incorporation of C_2 units into cholesterol. This factor, which was used in the present study, is in good agreement with the factor of 1.3 derived by Brunegraber

et. al. (1972). These workers perfused the rat liver in the presence of $^{3}H_2O$ and(^{14}C)-glucose and calculated the proportional glucose carbon contribution to the total rate of FA synthesis, allowing for discrimination against ^{3}H as described above. In the same perfusions, the ratio of C incorporation (calculated as C_2 units) to apparent H atom incorporation (assuming no discrimination against ^{3}H) into hepatic cholesterol was calculated. This ratio was consistently 1.31 x the glucose carbon contribution to total fatty acid synthesis at perfusate glucose concentrations of 8-25 mM. Thus, assuming a common precursor pool of acetyl CoA for both FA and cholesterol synthesis in the liver, the division of RTA for cholesterol synthesis by 1.68/1.31 = 1.3 would yield the true rate of incorporation of C_2 units into hepatic cholesterol.

The rate of ^{14}C-labelled substrate incorporation into lipid fractions was calculated in terms of :

$$\mu g \text{ atoms of C incorporated into lipid fraction} = \frac{^{14}C \text{ d.p.m. incorporated into lipid fraction per g of fresh liver.}}{\text{Initial specific radioactivity of } ^{14}C\text{-precursor in perfusate.}}$$

The incorporation of C_2 units of radioactive substrate into the lipid fraction is 0.5 X the above quotient. The initial specific radioactivity (d.p.m. / μg atom of C) was determined by dividing the perfusate ^{14}C (d.p.m. / ml) determined within 5 min. of the addition of isotope, by the concentration of ^{14}C-precursor in perfusate (μg atoms C / ml). It was shown that the specific radioactivity of circulating glucose in the present experiments remained relatively constant over the perfusion period,

thus the incorporation of glucose carbon into hepatic lipids has been expressed as true rates. The same applies to experiments using ^{14}C lactate where this substrate was not infused. However, in experiments where further unlabelled lactate was infused during the liver perfusion, the incorporation of (^{14}C)-lactate has been expressed as C_2 units of initial (^{14}C)-lactate incorporated in 1 h.

3 Results.

3.1 Control of Lipogenesis by Adrenal Glucocorticoid Hormones.

3.1.1 Fatty Acid Synthesis in Intact Rats.

3.1.2 Fatty Acid Synthesis in the Perfused Liver.

3.1.3 The Hepatic Synthesis of Mono-, Di- and Poly-enoic Fatty Acids.

3.1.4 The Distribution of de novo Synthesised Fatty Acids amongst Hepatic Glycerides.

3.1.5 Lipogenesis in Livers Perfused with Exogenous Fatty Acids.

3.1.6 Cholesterol Synthesis in the Perfused Liver.

3.1.7 The Restoration of Fatty Acid Synthesis in Adrenalectomised Rats by Cortisol Treatment in vivo.

3.1.8 The Effect of Cortisol in vitro upon Fatty Acid Synthesis in the Perfused Liver.

3.1.9 The Influence of Adrenalectomy upon Serum Insulin Concentration in the Rat.

3.2 The Hormonal Control of Hepatic Carbohydrate Metabolism.

3.2.1 The Control of Hepatic Glycogen Synthesis by Adrenal Steroids in Intact Fasted Rats.

3.2.2 The Effects of Adrenal Steroids and Vasopressin upon Glucose Release from the Perfused Livers of Fed Rats.

3.2.3 The Effects of Adrenal Steroids and Vasopressin upon Gluconeogenesis in the Perfused Livers of 48h. Fasted Rats.

3.3 Further Studies Concerning the Metabolic Actions of Arginine Vasopressin.

3.3.1 The Interaction between Vasopressin and Insulin in the Control of Hepatic Glycogenolysis.

3.3.2 The Influence of Vasopressin upon the Concentrations of Hepatic Metabolites.

3.3.3 The Influence of Vasopressin upon Hepatic Lipid Metabolism.

3.3.4 The Influence of Vasopressin and other Hormones upon Hepatic Concentrations of Adenosine-3',5'-cyclic Monophosphate.

3.3.5 The Influence of Glycogenolytic Hormones upon the Activity of Glycogen Phosphorylase in Intact Rats.

3.3.6 The Influence of Vasopressin and other Hormones upon Hepatic Concentrations of Guanosine-3',5'-cyclic Monophosphate.

3.3.7 The Role of Extracellular Ca^{++} in the Metabolic Actions of Vasopressin.

3 Results.

3.1 Control of Lipogenesis by Adrenal Glucocorticoid Hormones.

3.1.1 Fatty Acid Synthesis in Intact Rats.

The rates of hepatic fatty acid synthesis observed in intact rats remained unchanged following adrenalectomy (table 3), even in animals which were pretreated with an oral dose of glucose 30 min. prior to the determination of lipogenic rate. Hepatic cholesterol synthesis was similarly unchanged following adrenal ablation in these animals. In contrast, the total rate of fatty acid synthesis observed in the epididymal adipose tissue of adrenalectomized rats was lower than that of sham-operated controls. This impairment in fatty acid synthesis was most significant in the rats which received oral glucose pretreatment (table 3). In table 3 is also recorded the observation that the $^{14}C/^{3}H$ ratio in these experiments was consistently higher in adipose tissue than in liver. Assuming that (^{14}C)-glucose of the same specific radioactivity was available to all tissues at any one time during the experiment, this observation indicates that glucose plays only a relatively minor role as a precursor of hepatic fatty acids in the conditions tested.

Table 3 Synthesis of lipids in-vivo.

Table 3 Synthesis of lipids in vivo.

Male rats weighing 180 g. received $^{3}H_2O$ and (^{14}C)-glucose at 11.00 h. by intra-peritoneal injection. Samples of liver and epididymal adipose tissue were frozen in liquid nitrogen after 60 min. Some rats received glucose (2.0 ml, 2.0 M) intragastrically under light ether anaesthesia 30 min. prior to the injection of radioactive precursors. Other details are in the text. Results are means $\pm$ S.E.M. for the number of observations in parentheses. Differences between adrenalectomized and sham-operated or untreated rats within groups 1 and 2 are not significant unless indicated (*$P < 0.01$).

Experimental group	Season	Glucose treatment	μmol of C_2 units per g fresh wt. of tissue: Fatty acids: Liver	Fatty acids: Epididymal adipose tissue	Hepatic cholesterol	Ratio: R^1
1a.Adrenalectomized	Jan-Mar	-	7.1 ± 1.7(7)	3.5 ± 1.0(7)	6.4 ± 1.7 (6)	3.0 ± 1.1(7)
1b.Sham-operated	Jan-Mar	-	10.0 ± 1.6(7)	6.3 ± 1.8(6)	5.1 ± 1.7 (6)	4.5 ± 2.1(7)
1c.Untreated	Jan-Mar	-	9.4 ± 1.0(7)	10.5 ± 3.0(6)	6.8 ± 0.6 (5)	4.9 ± 1.4(6)
2a.Adrenalectomized	Sept-Oct	+	8.5 ± 2.2(10)	4.3 ± 1.1(10)*	3.7 ± 0.6 (8)	4.0 ± 1.2(10)
2b.Sham-operated	Sept-Oct	+	10.5 ± 2.0(12)	16.7 ± 3.8(12)*	3.0 ± 0.2 (8)	4.9 ± 0.7(11)

Abbreviation:

$$\text{Ratio } R^1 = \frac{^{14}C \text{ d.p.m/g}}{^{3}H \text{ d.p.m/g}} \text{ adipose} \Big/ \frac{^{14}C \text{ d.p.m/g}}{^{3}H \text{ d.p.m/g}} \text{ liver}$$

3.1.2 Fatty Acid Synthesis in the Perfused Liver.

The rates of fatty acid synthesis observed in the perfused livers of adrenalectomized rats were diminished compared with those of sham-operated controls or untreated rats (table 4). The perfusion conditions used in these experiments were chosen as a compromise between those known to permit maximal lipogenesis in the perfused mouse liver (Salmon et. al., 1974) and those which may exist in the hepatic portal vein following an oral glucose load (figure 7). Thus, the initial concentrations of glucose and lactate in these experiments were adjusted to 12 mM and 10 mM respectively. The resultant 'autoregulation' of glucose and lactate in the perfusate during the course of 100 min. perfusion is shown in figure 14. It is clear that during the period over which fatty acid synthesis was measured (40 - 100 min.), the glucose and lactate concentration of the perfusion medium remained approximately constant at 16 mM and 6 mM respectively.

The rates of fatty acid synthesis in perfused livers from adrenalectomized rats varied between 60 to 80% of those in sham-operated controls depending upon the season (table 4). There was a relative increase in all rates from November to April and during this period the diminution of fatty acid synthesis following adrenalectomy was less marked. There was a similar, but more marked diminution, in the release of newly synthesized fatty acids into the perfusate by livers of adrenalectomized rats.

The proportion of newly synthesized fatty acids derived from circulating glucose or lactate remained unaltered following adrenalectomy. Thus, in the perfused liver of the adrenalectomized

rat, fatty acid synthesis from lactate and glucose was impaired to the same extent as was the total rate. In the conditions of perfusion used in the present study lactate contributed about twice as many C atoms as glucose to total fatty acid synthesis.

In one experimental group, adrenalectomized rats were treated with a high dose of cortisol (100 mg kg^{-1}) two hours before perfusion. This group exhibited further diminution in fatty acid synthesis as compared with untreated adrenalectomized rats, and a particularly low rate of fatty acid synthesis from glucose.

It has been reported that many of the metabolic consequences of insulin lack may be ameliorated following adrenal ablation (see eg. Exton et. al., 1973). In experimental group 3 (table 4) the much reduced rate of fatty acid synthesis observed in streptozotocin diabetic rats remained unchanged if the animals were adrenalectomized 4 days after the injection of streptozotocin and 4 days before perfusion.

In livers perfused under these conditions fatty acids are synthesized mainly from glycogen and lactate, and to a lesser extent from glucose (Salmon et. al., 1974). In some of the experimental groups shown in table 4 the initial glycogen content of the liver was measured by sampling the right lobe 5 mins. before the addition of radioactive precursors to the perfusate. Within each experimental group, the initial hepatic glycogen content of the adrenalectomized rats did not differ significantly from that of the sham-operated controls. Thus, there was no apparent shortage of glycogen amongst the adrenalectomized animals.

Table 4 Synthesis of fatty acids in the perfused liver.

Table 4 Synthesis of fatty acids in the perfused liver.

Livers of fed rats were perfused as described in the text. $^{3}H_2O$ and ^{14}C labelled substrates were added after 40 min. and the radioactivity in fatty acids was determined after a further 60 min. Total fatty acid synthesis was calculated from ^{3}H incorporation. Glycogen was measured in the right lobe, removed after 35 min. perfusion. Other details are in the text. Results are means $\pm$ S.E.M. of the number of observations indicated. In each group, total fatty acid synthesis and export from the livers of adrenalectomized rats was significantly lower than that in sham-operated controls ($p < 0.01$, except in group 4, $p < 0.05$). Within group 3, 3e versus 3c or 3d, $p < 0.001$, 3c versus 3d N.S., 3b versus 3e N.S.

	Experimental group	Season	^{14}C-labelled precursor	Total fatty acid synthesized (μmol C_2-units/h per g of liver)		Percent from ^{14}C-labelled precursor		Initial glycogen (μmol of glucose/g fresh wt. of liver)
				liver	perfusate	liver	perfusate	
1a	Adrenalectomized (9)	Nov-Jan	Glucose	20.5 ± 0.9	2.1 ± 0.2	18 ± 1	18 ± 1	—
1b	Sham-operated (8)	Nov-Jan	Glucose	28.7 ± 2.2	5.3 ± 0.9	19 ± 1	19 ± 1	—
1c	Adrenalectomized treated with cortisol* (6)	Nov-Jan	Glucose	11.0 ± 1.9	2.1 ± 0.6	10 ± 2	10 ± 2	—
2a	Adrenalectomized (4)	April	Glucose	19.2 ± 3.1	1.6 ± 0.1	13 ± 1	14 ± 1	—
2b	Sham-operated (6)	April	Glucose	24.5 ± 1.0	3.3 ± 0.3	18 ± 2	16 ± 1	—
3a	Adrenalectomized (6)	July	Glucose	11.3 ± 1.4	0.7 ± 0.3	14 ± 1	22 ± 2	281 ± 44
3b	Sham-operated (6)	July	Glucose	18.8 ± 1.8	2.4 ± 0.2	17 ± 1	19 ± 2	339 ± 27
3c	Streptozotocin-diabetic (4)	July	Glucose	3.7 ± 1.1	0.5 ± 0.1	6 ± 1	—	51 ± 15
3d	Streptozotocin-diabetic/ adrenalectomized (4)	July	Glucose	3.8 ± 0.9	0.5 ± 0.1	7 ± 1	—	47 ± 5
3e	Untreated (5)	July	Glucose	22.3 ± 1.5	3.4 ± 1.1	20 ± 1	19 ± 2	352 ± 28
4a	Adrenalectomized (3)	Sept	Lactate	12.6 ± 2.7	1.4 ± 0.4	28 ± 4	42 ± 10	302 ± 16
4b	Sham-operated (4)	Sept	Lactate	21.4 ± 1.3	2.9 ± 0.4	29 ± 3	32 ± 8	332 ± 12

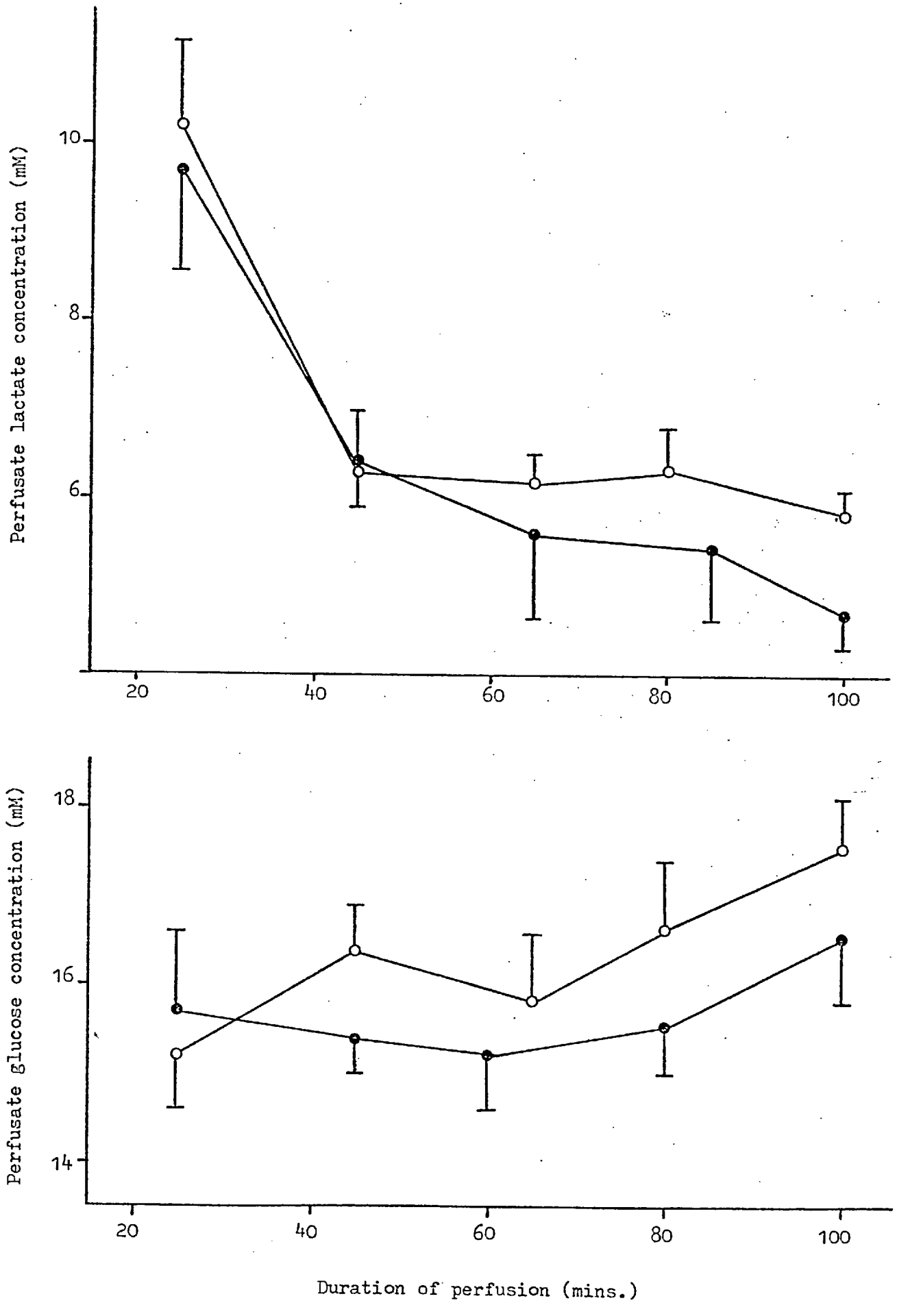
* Rats received cortisol <u>in vivo</u> (100 mg/kg) 2 h. before perfusion

Fig. 14 The concentration of glucose and lactate in perfusion medium.

Fig. 14 The concentration of glucose and lactate in perfusion medium.

Experiments designed to study lipogenesis in perfused rat livers were performed as described in the text. Glucose and lactate were added to the perfusion medium to initial concentrations of 12 mM and 10 mM respectively. Fatty acid synthesis was measured between 40 and 100 mins. of perfusion during which time the concentrations of glucose and lactate remained approximately constant.

Results are means ± S.E.M. of at least 6 sham-operated (○), and at least 7 adrenalectomized rats (●).


Perfusate lactate concentration (mM)
10
8
6
20
40
60
80
100
Perfusate glucose concentration (mM)
18
16
14
20
40
60
80
100
Duration of perfusion (mins.)

3.1.3 The Hepatic Synthesis of Mono-, Di- and Poly-enoic Fatty Acids.

The separation by t.l.c., of total hepatic fatty acids from perfused livers according to their degree of unsaturation (table 5), demonstrated that the synthesis of monoenoates was particularly diminished following adrenalectomy. Thus, monoenoic fatty acids contributed about 5% to the total of newly synthesized fatty acids in the perfused livers of adrenalectomized rats, whilst in sham-operated rats these fatty acids made up 11% of the total. There was a corresponding increase in the contribution of saturated fatty acids to the total synthesized in the adrenalectomized group.

The subcutaneous injection of cortisol (2 mg. kg^{-1}) into adrenalectomized rats (5.2 hr. before the mid-point of fatty acid synthesis determination during perfusion) resulted in a restoration in the rate of monoenoate synthesis (table 5). This pretreatment also gave rise to a further increase in the rate of synthesis of di - and poly-enoic fatty acids and a corresponding decrease in the contribution of saturated fatty acids to the total rate.

Table 5 The distribution of ^{3}H and ^{14}C in de novo synthesized fatty acids, separated according to their degree of saturation.

Table 5 The distribution of ^{3}H and ^{14}C in de novo synthesized fatty acids separated according to their degree of saturation.

Livers from fed rats were perfused (in April) with $^{3}H_2O$ and (^{14}C)-glucose as described in table 4 or after treatment (in vivo) with cortisol as in figure 15. After direct saponification of liver samples, fatty acid methyl esters were prepared as described in the text and separated according to their degree of saturation by t.l.c. Data are expressed as a percentage of the radioactivity recovered from the entire t.l.c. plate, which was at least 90% of that detected in total fatty acids after direct saponification. Other details are in the text. Results are means $\pm$ S.E.M. of 3 measurements in adrenalectomized rats and 4 in sham-operated controls. Differences between groups are significant as indicated.

Experimental group	Total fatty acid synthesis (umol of C_2 units/h per g)	Radioactivity in fatty acid (%)					
		Saturated		Monoenoic		Di- and poly-enoic	
		^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
1.Adrenalectomized	18.3 ± 3.6	90.3 ± 0.3	85.5 ± 0.1	4.1 ± 0.2	5.4 ± 0.2	5.6 ± 0.1	6.3 ± 0.1
2.Sham-operated	26.9 ± 1.3	85.2 ± 0.8	84.8 ± 0.3	10.8 ± 0.8	11.3 ± 0.7	4.1 ± 0.7	3.8 ± 0.6
3.Adrenalectomized, given cortisol 5.2h before perfusion.	28.7 ± 2.6	81.9 ± 1.0	80.3 ± 1.3	9.9 ± 2.5	11.5 ± 2.4	8.2 ± 1.4	8.2 ± 1.3
		P values					
1 versus 2	<0.02	<0.01	<0.001	<0.001	<0.001	N.S	<0.02
2 versus 3	N.S.	N.S.	<0.01	N.S.	N.S.	<0.02	<0.02
1 versus 3	N.S.	<0.001	<0.01	N.S.	<0.05	N.S.	N.S.

3.1.4 The Distribution of de novo Synthesized Fatty Acids amongst Hepatic Glycerides.

The separation of lipid classes by thin layer chromatography indicated that the synthesis of both phospholipid fatty acids and triacylglycerol fatty acids was inhibited following adrenalectomy (table 6). However, the synthesis of triacylglycerol fatty acid was diminished to a greater extent than was that of phospholipid fatty acids. This is particularly clear from the ratio:d.p.m. in triacylglycerol fatty acid / d.p.m. in phospholipid fatty acid shown in table 6. There was a similar diminution in the rate of fatty acid synthesis from glucose in these two lipid classes as was observed in the total rate.

Thin layer chromatography was also used to study the nature of the inhibition of hepatic fatty acid synthesis in streptozotocin diabetes. Streptozotocin diabetic rats exhibited a similar reduction in the de novo synthesis of fatty acids in these two lipid classes as did adrenalectomized rats. However, in the perfused livers of streptozotocin diabetic rats the inhibition of fatty acid synthesis in both lipid classes was more marked. Also, while total triacylglycerol fatty acid synthesis was inhibited to a significantly greater extent than was the synthesis of phospholipid fatty acids, the glucose carbon contribution to fatty acid synthesis for both lipid classes was diminished to a similar extent following the induction of streptozotocin diabetes.

Perfusate glycerides were also separated by thin layer chromatography. 75 - 85% of the radioactivity in newly synthesized fatty acids released into the perfusion medium was found

in triacylglycerols and no significant alteration in this proportion was observed in the perfusate from livers following adrenalectomy, (results not shown).

Table 6 The distribution of de novo synthesized fatty acids amongst liver glycerides.

Table 6 The distribution of de novo synthesized fatty acids amongst liver glycerides.

In some groups of livers described in table 4, triacylglycerol and phospholipid were separated by thin layer chromatography as described in the text. After elution from their various bands, the glycerides were saponified and the radioactivity in fatty acids determined by liquid scintillation spectrometry. During this procedure, about 9% of radioactivity in fatty acids was lost during washing (Folch et. al., 1957) and another 5% during thin layer chromatography and elution procedures. Data are not corrected for these losses. Other details are in the text. Results are means $\pm$ S.E.M. for the number of observations indicated in parentheses. For each season the differences between experimental and control groups are highly significant ($p < 0.01$) unless otherwise indicated (*).

Experimental Group	Season	Total fatty acid synthesis (μmol of C_2 units/h per g)			Fatty acid synthesis from glucose (%)			Ratio: $\frac{\text{d.p.m. in TGFA}}{\text{d.p.m. in PLFA}}$	
		All classes (no Folch extraction)	TGFA	PLFA	All classes (no Folch extraction)	TGFA	PLFA	^3H	^{14}C
Adrenalectomized(7)	Nov-Dec	19.7 ± 0.9	4.8±0.7	9.2±0.8	20 ± 3*	19±2*	17±2*	0.52±0.04	0.57±0.05
Sham-operated (4)	Nov-Dec	32.0 ± 2.4	13.3±1.2	13.1±0.4	22 ± 3*	27±2*	24±2*	1.09±0.11	1.19±0.12
Normal (4)	July	29.8 ± 8.0	14.2±1.6	12.5±1.2	14 ± 1	15±1	13±1	1.18±0.17	1.34±0.20*
Streptozotocin-diabetic (4)	July	3.5 ± 0.6	0.8±0.2	1.5±0.3	3.1 ± 0.1	5.9±1.9	2.5±0.4	0.50±0.10	0.97±0.17*

Abbreviations:

TGFA = Triacylglycerol fatty acid.

PLFA = Phospholipid fatty acid.

3.1.5 Lipogenesis in Livers Perfused with Exogenous Fatty Acids.

The preferential inhibition of triacylglycerol synthesis suggested that the reduced rate of hepatic fatty acid synthesis observed in the perfused livers of adrenalectomized rats could be the consequence of an inhibition of the processes whereby fatty acids are incorporated into triacylglycerols. This possibility was tested in perfusions in which albumin-bound ^{14}C-labelled fatty acids were infused into the perfusate in order to maintain a constant concentration of circulating fatty acid (table 7).

The incorporation of (^{14}C)-oleate into hepatic glycerides was not altered following adrenalectomy (table 7). The total rate of de novo fatty acid synthesis in sham-operated rats was less than in the absence of oleate, reflecting the inhibition of de novo synthesis by oleate (Mayes and Topping, 1974). This reduced rate of de novo synthesis was similar to that observed in the livers of adrenalectomized rats perfused with oleate; thus the relative impairment of fatty acid synthesis in the livers of adrenalectomized rats was not discernible in these perfusions with oleate.

In the presence of circulating (^{14}C)-palmitate, the incorporation of this fatty acid into liver glycerides was also unaltered following adrenalectomy. In these experiments however, the inhibition of de novo fatty acid synthesis (measured with $^{3}H_2O$) by circulating palmitate was more marked than the corresponding inhibition with oleate. Also, in contrast to the results of the experiments with circulating oleate, the presence of palmitate in the perfusion medium did not alter the relatively greater

inhibition of de novo fatty acid synthesis in the adrenalectomized group.

These two circulating fatty acids also exerted differing effects upon the relatively greater inhibition of triacylglycerol fatty acid synthesis de novo previously observed in the perfused liver of the adrenalectomized rat. In the perfusions with (^{14}C)-oleate, there was still a relative impairment in the de novo synthesis of triacylglycerol fatty acids as compared with phospholipid fatty acids following adrenalectomy. However, when (^{14}C)-palmitate was the circulating fatty acid, the ratio of ^{3}H d.p.m. in triacylglycerol fatty acid / ^{3}H d.p.m. in phospholipid fatty acid was larger than the corresponding ratio in the absence of exogenous fatty acid, and similar in both adrenalectomized and sham-operated groups.

Table 7 Lipogenesis in livers perfused with circulating fatty acids.

Table 7 Lipogenesis in livers perfused with circulating fatty acids.

Livers were perfused (during March - May) as described in the text. Albumin-bound ^{14}C-labelled fatty acids were added to the perfusate after 40 min. perfusion, and infused to maintain a constant concentration and specific radioactivity for a further 30 mins, after which time liver samples were taken. De novo incorporation of C_2 units into hepatic fatty acids was measured with $^{3}H_2O$. Lipid classes were separated by t.l.c. prior to saponification as described in the text. The values for the incorporation of precursors into total hepatic fatty acids were derived from directly saponified liver samples. Results are means $\pm$ S.E.M. of at least four rats in each group. The differences between various groups are significant as indicated.

Experimental group	Circulating fatty acid	Conversion of precursors into hepatic glycerides. (μmol/h per g of fresh liver): Exogenous fatty acid			C_2 units			Ratio: d.p.m. in TGFA / d.p.m. in PLFA	
		Total	TGFA	PLFA	Total	TGFA	PLFA	^{14}C	^{3}H
1. Adrenalectomized	0.7mM Oleate	18.1±2.9	7.6±1.4	5.0±0.9	16.4±1.3	4.2±0.6	5.6±0.8	1.52±0.08	0.75±0.05
2. Sham-operated	0.7mM Oleate	18.0±1.9	7.2±1.1	4.1±0.6	15.9±0.7	5.0±0.7	4.6±0.7	1.77±0.18	1.07±0.07
3. Sham-operated	-	-	-	-	24.6±1.0	7.3±1.0	8.7±1.0	-	1.07±0.36
4. Adrenalectomized	0.95mM Palmitate	15.6±2.9	8.4±1.2	2.5±0.4	3.1±0.4	1.1±0.2	0.5±0.1	3.6 ±0.6	2.4 ±0.3
5. Sham-operated	0.95mM Palmitate	17.3±3.9	10.7±2.3	2.7±0.1	7.4±1.2	4.0±0.6	1.4±0.2	3.9 ±0.2	2.8 ±0.3
		P values							
1 versus 2		N.S	N.S	N.S	N.S	N.S	N.S	N.S	<0.01
1 versus 3		-	-	-	<0.01	<0.05	<0.02	-	N.S
2 versus 3		-	-	-	<0.001	<0.01	<0.05	-	N.S
4 versus 5		N.S	N.S	N.S	<0.01	<0.01	<0.01	N.S	N.S
4 versus 3		-	-	-	<0.001	<0.001	<0.001	-	<0.05
5 versus 3		-	-	-	<0.001	<0.1	<0.01	-	<0.02

Abbreviations:

TGFA = Triacylglycerol fatty acid.
PLFA = Phospholipid fatty acid.

3.1.6 Cholesterol Synthesis in the Perfused Liver.

In some perfusions, cholesterol synthesis was measured by ^{3}H incorporation from $^{3}H_2O$ and ^{14}C incorporation from (^{14}C)-glucose. There was no consistently discernible significant alteration in cholesterol synthesis following adrenalectomy in livers perfused at two different seasons (table 8). There was also no significant decrease in total cholesterol synthesis in the perfused livers of streptozotocin diabetic rats as compared with citrate injected controls. The contribution of glucose-carbon to total cholesterol synthesis was much reduced in diabetic rats, presumably reflecting the diminished hepatic glucose uptake in these animals.

Table 8. Cholesterol synthesis in the perfused liver.

In some of the perfusions described in Tables 4 and 5, cholesterol synthesis was followed by the incorporation of ^{3}H from $^{3}H_2O$ and ^{14}C from (^{14}C)-glucose. For other details, see the text. Results are means ± S.E.M. for the numbers of observations indicated.

Experimental group		Season	No. of perfusions	Cholesterol synthesis (umol of C_2 units/h per g fresh wt. of liver)	
				Total	From glucose
1a.	Adrenalectomized	Nov-Jan	8	4.1 ± 0.5 ‡	0.82 ± 0.13 ‡
1b.	Sham-operated	Nov-Jan	10	2.5 ± 0.3 ‡	0.43 ± 0.06 ‡
1c.	Adrenalectomized*	Nov-Jan	6	2.4 ± 0.6	0.19 ± 0.05
2a.	Adrenalectomized	April	3	3.0 ± 0.5	0.36 ± 0.03
2b.	Sham-operated	April	3	2.6 ± 0.3	0.32 ± 0.06
3a.	Diabetic	July	4	2.3 ± 0.7	0.08 ± 0.04 †
3b.	Normal	July	4	3.2 ± 0.6	0.35 ± 0.04 †

* Treated for 2 h. in vivo with 100 mg of cortisol/kg.

‡ $P < 0.02$, group 1a versus 1b.

† $P < 0.001$.

3.1.7 The Restoration of Fatty Acid Synthesis in Adrenalectomized Rats by Cortisol Treatment in vivo.

The ability of cortisol to restore fatty acid synthesis to normal in the perfused livers of adrenalectomized rats was tested at two different doses, selected after a consideration of earlier studies (e.g. Klausner and Heimberg, 1967).

Adrenalectomized rats, injected subcutaneously with a large dose of cortisol (100 mg. kg^{-1}) 2 hours prior to the commencement of perfusion (i.e. 3.2 hr. before the mid-point of fatty acid synthesis determination), exhibited a further diminution in fatty acid synthesis as compared with untreated adrenalectomized rats (table 4). These pretreated rats also exhibited a particularly low rate of fatty acid synthesis from glucose.

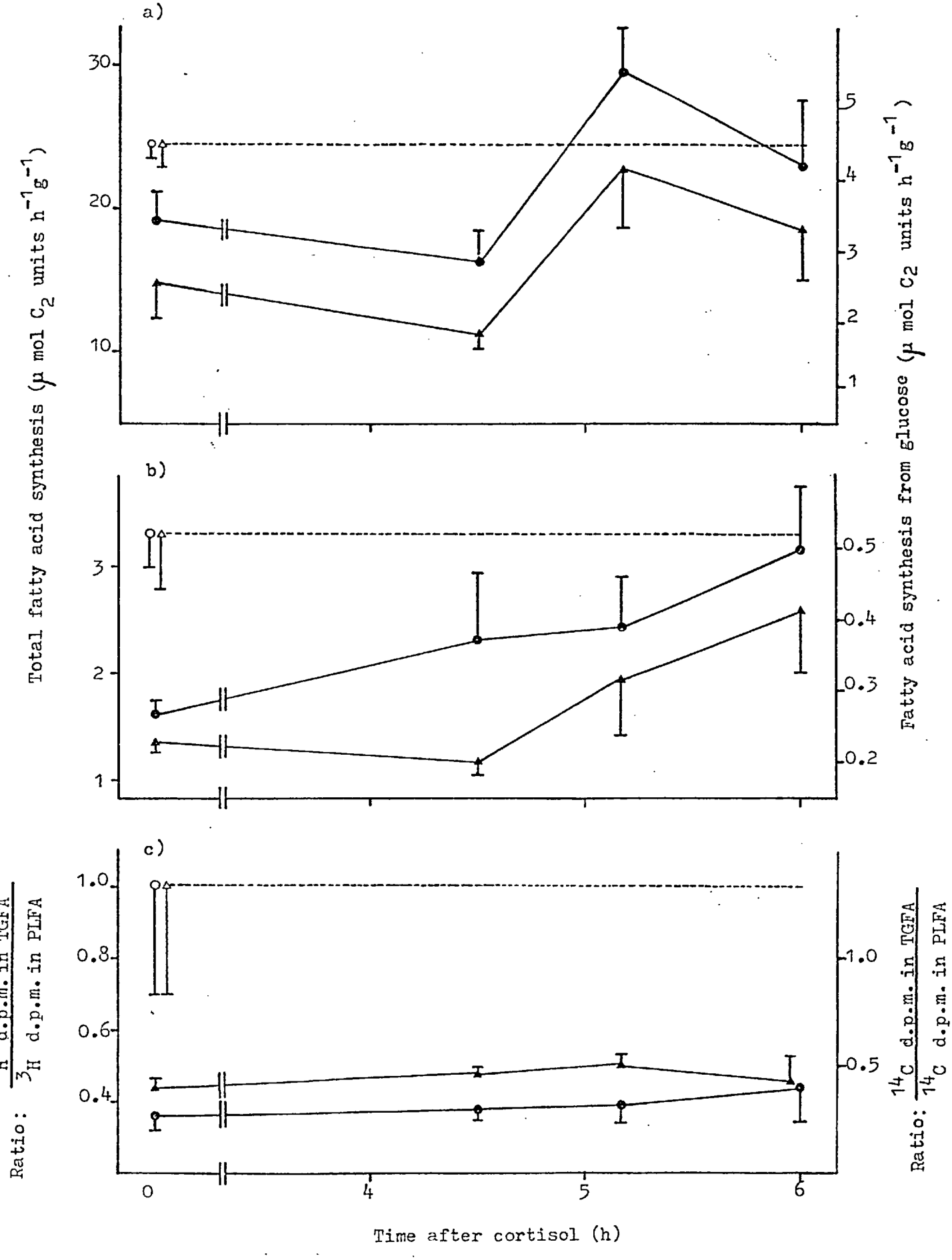
Similar pretreatment of adrenalectomized rats in vivo with a lower dose of cortisol (10 mg. kg^{-1}) restored the capacity for fatty acid synthesis in the subsequently perfused liver within 4-5 hr. (figure 15a). The release of newly-synthesized fatty acid from the perfused liver was restored to normal after one further hours pretreatment (figure 15b).

In figure 15c is shown the ratio:radioactivity in triacylglycerol fatty acid / radioactivity in phospholipid fatty acid, at various times after subcutaneous cortisol injection (10 mg. kg^{-1}) in vivo. It is clear that while this pretreatment was effective in restoring total hepatic fatty acid synthesis to normal in the perfused livers of adrenalectomized rats, the relative impairment of triacylglycerol fatty acid synthesis remained unchanged.

Fig. 15 The restoration of hepatic fatty acid synthesis following cortisol treatment in vivo.

Fig. 15 The restoration of hepatic fatty acid synthesis following cortisol treatment in vivo.

Adrenalectomized rats were treated with cortisol (10 mg. kg^{-1} subcutaneously) and their livers were then perfused as described in the text. Fatty acid synthesis was measured between 40 and 100 mins. of perfusion and the pretreatment times shown in the figure were calculated from the time of cortisol injection (in vivo) to 70 mins. after the start of perfusion. The incorporation of C_2 units into hepatic fatty acids (a) was measured as a total rate (●) and from glucose (▲). Export of newly synthesized fatty acids (b) was similarly measured as a total rate (●) and as export of fatty acids synthesized from glucose (▲). In (c) are shown the ratios of the radioactivity incorporated into the fatty acids of triglycerides, to that incorporated into phospholipid fatty acids. (●) denotes the ratio for ^{3}H incorporation, while (▲) shows the corresponding ratio for ^{14}C incorporation. For each group, the value at zero time refers to untreated adrenalectomized rats and the corresponding open symbol and broken line denotes the values obtained with sham-operated animals. Results are means $\pm$ S.E.M. from at least 3 perfusions in each group.


a)
b)
c)
Total fatty acid synthesis (μ mol C_2 units $h^{-1}g^{-1}$)
Fatty acid synthesis from glucose (μ mol C_2 units $h^{-1}g^{-1}$)
Ratio: $\frac{^3H \text{ d.p.m. in TGFA}}{^3H \text{ d.p.m. in PLFA}}$
Ratio: $\frac{^{14}C \text{ d.p.m. in TGFA}}{^{14}C \text{ d.p.m. in PLFA}}$
Time after cortisol (h)

3.1.8 The Effect of Cortisol in vitro upon Fatty Acid Synthesis in the Perfused Liver.

The results of the experiments described in figure 15 indicated that the reduced rate of fatty acid synthesis in the perfused livers of adrenalectomized rats could be restored to control levels by cortisol pretreatment in vivo. These results raised the question of whether cortisol acts directly on the liver to produce such a restoration. The results in figure 15 indicated that long perfusions would be necessary in order to demonstrate any such effects. Therefore, perfusions lasting 5½ hr. were performed in which cortisol was present throughout perfusion, (infused to maintain a constant concentration : table 9). The rate of fatty acid synthesis was measured over the last hour of perfusion and the expected diminution in the synthesis and release of newly synthesized fatty acids was observed following adrenalectomy. There was no discernible restoration of synthesis or release of fatty acids in the livers of adrenalectomised rats perfused in the presence of cortisol.

Table 9. Effect of cortisol on fatty acid synthesis in the perfused liver.

Livers from fed rats were perfused (in September) for 5.5 h. with 100 ml of medium containing glucose (12 mM) and lactate (5 mM). In one group the perfusate also contained cortisol (10 μg/ml initially, and then infused at 1.6 mg/h). After 4.5 h. perfusion $^{3}H_2O$ and (^{14}C)-glucose were added. The radioactivity in total fatty acids was determined after a further 60 min. Other details were as described in Table 4. Results are means ± S.E.M. for the number of observations indicated. For all parameters, values in group 3 are not significantly different from those in group 1.

Experimental group	Number of perfusions	Fatty acid synthesized (μmol of C_2 units/h per g)		Fatty acid synthesized from glucose (%)	
		Liver	Perfusate	Liver	Perfusate
1. Adrenalectomized	3	12.4 ± 2.6	2.4 ± 0.6	9.6 ± 1.5	15.5 ± 1.5
2. Sham-operated	3	19.4 ± 3.4	5.3 ± 1.0	11.8 ± 0.4	8.0 ± 2.1
3. Adrenalectomized, plus cortisol in perfusate.	4	10.0 ± 2.0	1.7 ± 0.1	8.7 ± 1.6	13.1 ± 1.6

3.1.9 The Influence of Adrenalectomy upon Serum Insulin Concentration in the Rat.

It is clear from the results described in this section that adrenal ablation results in a consistently discernible diminution of hepatic fatty acid synthesis. The synthesis of monoenoic fatty acids was particularly affected following adrenalectomy, as was the export of newly synthesized fatty acids from the liver. As will be discussed in detail in section 4.1.6, these effects of adrenalectomy are similar to those which occur as a result of insulin lack. This fact, together with the observation that steroid treatment in vivo resulted in a restoration of lipogenic capacity in the perfused livers of adrenalectomized rats, suggested that the assay of serum insulin in adrenalectomized, sham-operated and normal rats might be of interest.

In table 10 is shown the results of such an assay which also included serum from normal fed rats which had been given 0.9% NaCl in their drinking water for 7 days prior to sacrifice (as had the adrenalectomized and sham-operated groups). The serum insulin concentration of the adrenalectomized group was significantly lower than that of the sham-operated controls ($p < 0.02$), but NaCl treatment did not appear to influence the concentration of the hormone in normal rat serum.

Table 10 The influence of adrenalectomy and sham-operation upon serum insulin concentration in the rat.

Fed 180 g. rats were adrenalectomized, sham-operated or given 0.9% NaCl in their drinking water 7 days before sacrifice. Untreated animals, and those described above, were killed by cervical fracture and 1 ml. blood samples were taken by aortic puncture. Serum insulin was measured by radioimmunoassay using a commercial rat insulin standard (assays kindly performed by Mr. C. Hardcastle). Results are means ± S.E.M. of the number of observations indicated. The difference between groups 1 and 2 is significant ($p < 0.02$), all other differences are not.

Experimental group	No. of observations	Serum insulin concentration ($\mu U. ml^{-1}$)
1. Adrenalectomized	4	72 ± 12
2. Sham-operated	4	148 ± 22
3. Normal	4	117 ± 17
4. Normal - NaCl treated	4	114 ± 47

3.2 The Hormonal Control of Hepatic Carbohydrate Metabolism.

The results of the first section revealed a marked diminution in lipogenic capacity following adrenalectomy in the rat. However, the experiments using ^{14}C-labelled precursors did not reveal any specific impairment of the incorporation of a particular precursor into hepatic fatty acids following adrenal ablation. Fatty acid synthesis consumes carbohydrate precursors, and under the conditions of perfusion described in the last section, the principal precursors of lipogenesis are likely to be glucose, lactate and especially hepatic glycogen (Salmon et. al. 1974). Hence, it was of interest to study the role of the adrenal gland in the control of hepatic glycogen metabolism.

3.2.1 The Control of Hepatic Glycogen Synthesis by Adrenal Steroids in Intact Fasted Rats.

Glycogen synthesis, measured in intact 48 h. fasted rats with a continuous tail vein infusion of glucose (3 m mol.h^{-1}), was found to be considerably reduced following adrenal ablation (table 11). The rates of glycogen synthesis measured in sham-operated control rats were similar to those recorded in un-operated normal animals.

In these experiments, the initial glycogen content of the liver (in a sample taken 10 min. after the commencement of glucose infusion) was about 40 - 50 μ mol glucose. g^{-1} in both sham-operated and un-operated rats. In contrast, the initial hepatic glycogen content of fasted adrenalectomized rats was about

2 μ mol glucose. g^{-1}.

When the starved adrenalectomized rats were pretreated with 2 ml. of 1.5 M glucose(delivered into the stomach via a small stomach tube 90 mins. before the commencement of tail vein infusion),the measured rate of hepatic glycogen synthesis was partially restored towards the control value. The initial hepatic glycogen content of these pretreated adrenalectomized rats was still significantly lower than that of the non-pretreated control animals. When sham-operated control rats were pretreated with intra-gastric glucose,their measured rate of hepatic glycogen accumulation was unaltered, but their initial hepatic glycogen levels increased $2\frac{1}{2}$ fold.

Table 11 Hepatic glycogen synthesis in intact 48 h. fasted rats.

Table 11 Hepatic glycogen synthesis in intact 48 h. fasted rats.

Rats were fasted for 48 h. and anaesthetized with nembutal, 1 M glucose was infused into the bloodstream via a cannula inserted in the tail vein as described in the text. Serial liver samples were taken 10 mins. and 70 mins. after the commencement of glucose infusion and the glycogen content estimated as previously described. Two groups of rats were pretreated with 2 ml 1.5 M glucose (administered intra- gastrically (I.G.) via a stomach tube) 90 mins. before the tail vein cannulation. Results are means $\pm$ S.E.M. of the number of observations indicated. Other details are in the text.

Experimental group	No. of observations	Pretreatment	Final blood glucose concentration (mM)	Initial hepatic glycogen content (μ mol glucose g^{-1})	Rate of hepatic glycogen synthesis (μ mol glucose $g^{-1}.h^{-1}$)
1. Normal	6	—	40.7 ± 4.6	42.0 ± 8.5	0.65 ± 0.06
2. Adrenalectomized	4	—	34.2 ± 1.5	2.1 ± 0.4	0.03 ± 0.02
3. Sham-operated	6	—	32.4 ± 5.6	53.6 ± 7.8	0.63 ± 0.09
4. Adrenalectomized	4	2 mls 1.5 M glucose 1.G.	36.1 ± 1.8	16.2 ± 5.3	0.25 ± 0.06
5. Sham-operated	3	2 mls 1.5 M glucose 1.G.	26.0 ± 6.7	138.1 ± 9.9	0.57 ± 0.15
				P values	
1 versus 3			N.S.	N.S.	N.S.
2 versus 3			N.S.	<0.001	<0.001
2 versus 4			N.S.	<0.05	<0.02
3 versus 4			N.S.	<0.01	<0.02
3 versus 5			N.S.	<0.001	N.S.

3.2.2 The Effects of Adrenal Steroids and Vasopressin upon Glucose Release from the Perfused Livers of Fed Rats.

Having established a role for the adrenal gland in the maintenance of normal hepatic glycogen synthesis in the starved rat, it was of interest to study the influence of adrenocorticoids upon hepatic glycogen breakdown. This follows because the degradation of liver glycogen is a major source of carbon for hepatic lipogenesis.

It has been demonstrated by a number of workers that adrenal corticosteroids are essential co-factors for the stimulation of hepatic glycogen degradation by glucagon and adrenalin (Exton et. al., 1972a). In the present study, experiments were designed to investigate the role of adrenal corticosteroids in the glycogen breakdown induced in the perfused liver by the hormone 8-arginine vasopressin (Hems and Whitton, 1973).

Livers of fed rats perfused with an initial concentration of 5 mM glucose in the perfusate soon establish a steady circulating glucose concentration of about 8 - 10 mM. A single dose of (8-arginine)-vasopressin (50 - 500 $\mu U.ml^{-1}$) resulted in a rapid release of glucose from the liver into the perfusion medium, and a corresponding decrease in hepatic glycogen concentration (figure 16). Glucose release into the perfusion medium accounted for about 60 - 70% of the hepatic glycogen breakdown when vasopressin was added to the perfusion medium. 40 mins. after the addition of the hormone to the perfusion medium, the concentration of glucose was generally reaching a steady state in the perfusate. in subsequent experiments the amount of glucose released by

the liver over this 40 minute period was taken as an index of the glycogenolytic potency of the hormone addition.

A study of the dose dependence of vasopressin-induced glucose release from the perfused liver (figure 17) revealed that this process was diminshed following adrenalectomy in response to low circulating concentrations of the hormone. Thus, whilst vasopressin-induced hepatic glucose output was maximal at a hormone dose of 500 $\mu U.ml^{-1}$ in sham-operated controls, the amount of glucose released by the perfused livers of adrenalectomized rats continued to rise up to a hormone dose of 1 $mU.ml^{-1}$. It is unlikely that reduced hepatic glycogen concentrations in adrenalectomized rats were responsible for this diminished vasopressin-induced glucose release. This follows from the results of the experiments reported in table 4, the perfused livers of adrenalectomized rats did not have significantly lower hepatic glycogen levels than those of sham-operated controls.

When adrenalectomized rats were injected sub-cutaneously with cortisol (10 $mg.kg^{-1}$: 4 hours prior to perfusion), vasopressin-induced glucose release from the subsequently perfused liver was restored towards the control level.

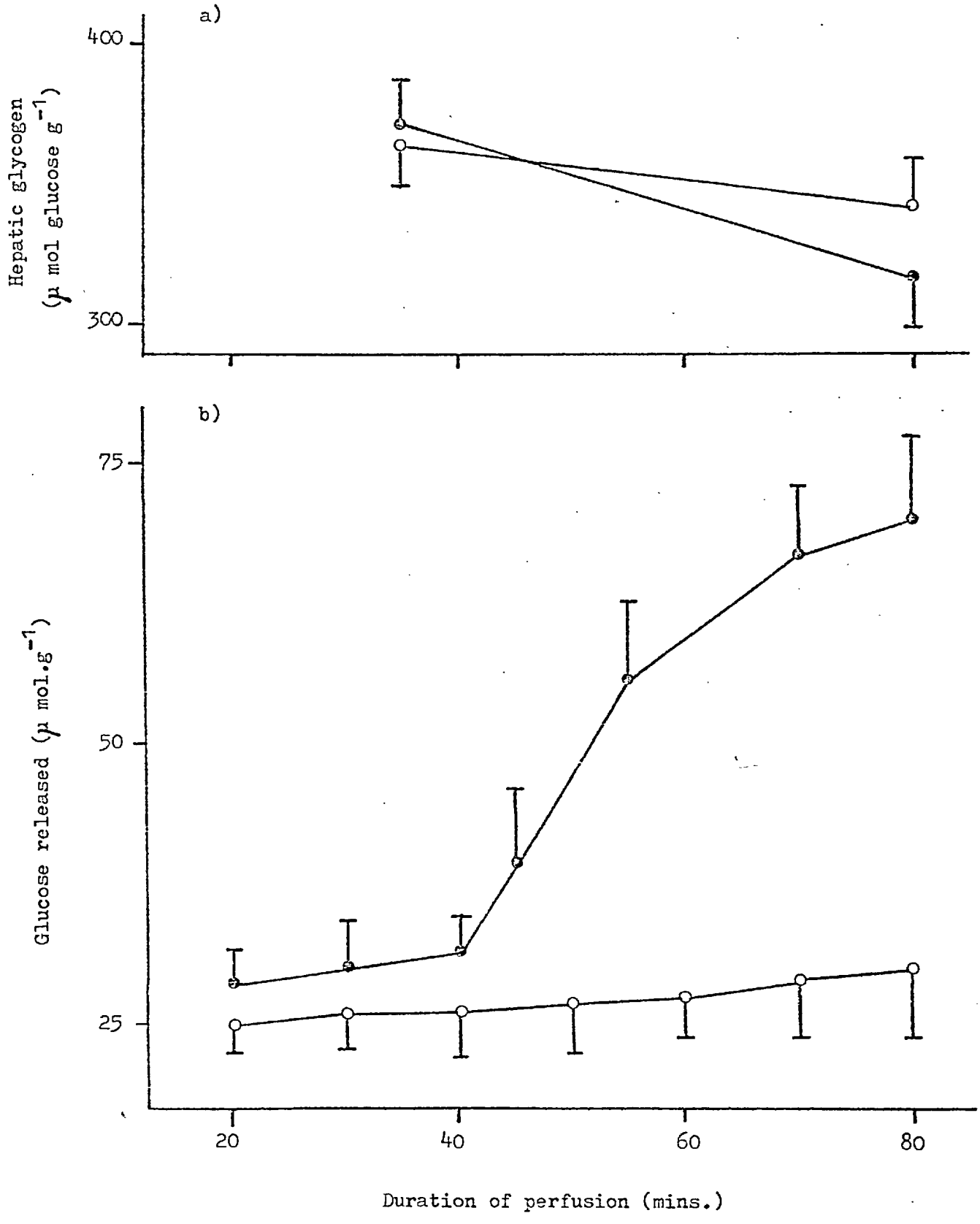
Fig. 16 The effect of vasopressin upon glucose releaseby, and glycogen content of, the perfused liver.

Fig. 16 The effect of vasopressin upon glucose release by and glycogen content of the perfused liver.

Livers from fed rats were perfused as described in the text. After 40 mins. vasopressin was added to some perfusions to an initial concentration of 400 $\mu U. ml^{-1}$. Serial liver samples were taken at 35 and 80 mins. for the determination of hepatic glycogen content (graph a).

Glucose was assayed in samples of perfusion medium removed from the collecting vessel at the times indicated. Thus, the glucose released during the perfusion was calculated (graph b).

Results are means $\pm$ S.E.M. of 3 control perfusions (O) and three perfusions with vasopressin (●).


a)
400
300
Hepatic glycogen
(μ mol glucose g⁻¹)
b)
75
50
25
Glucose released (μ mol.g⁻¹)
20
40
60
80
Duration of perfusion (mins.)

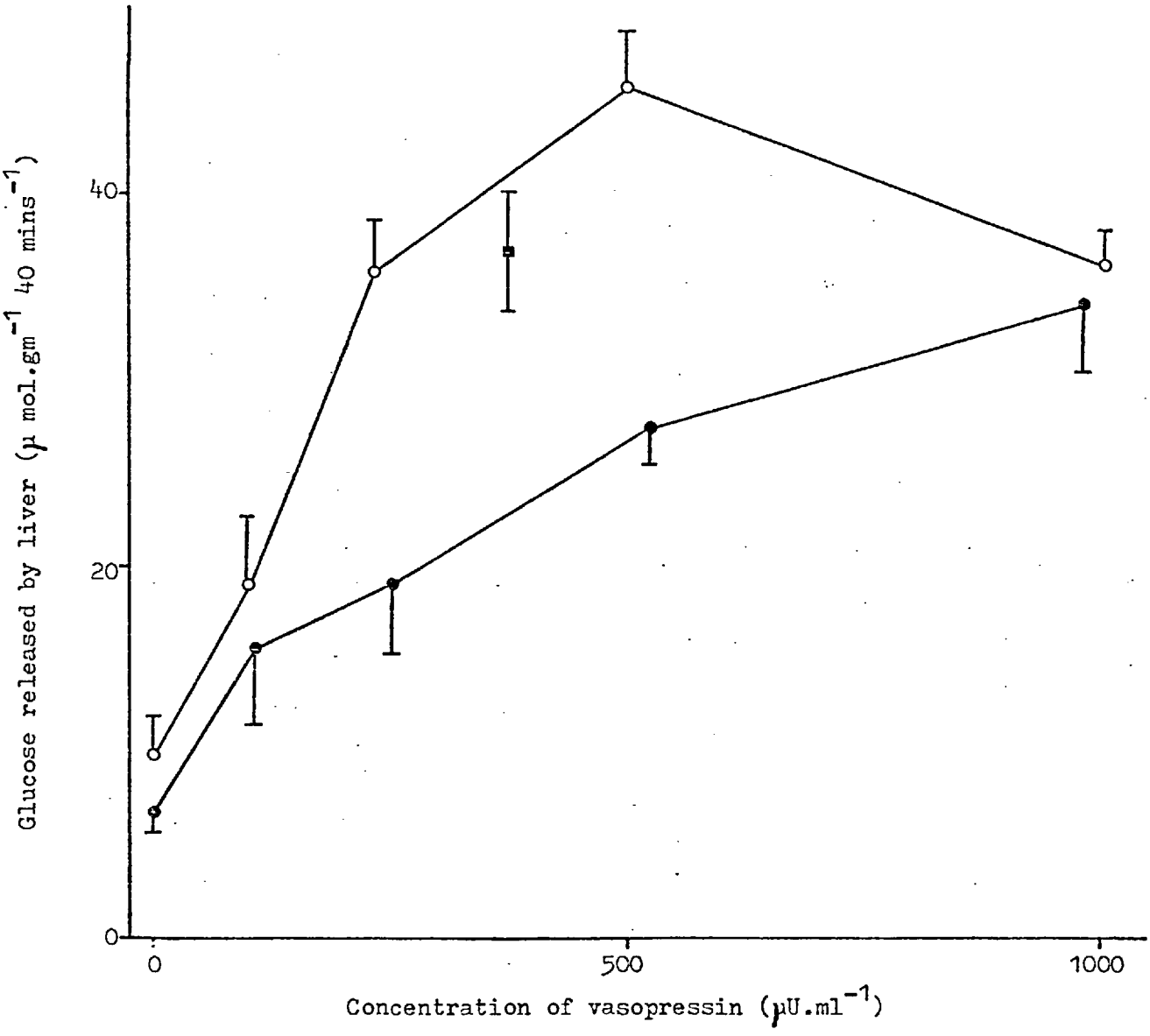


Fig. 17 The effect of adrenalectomy and steroid replacement upon vasopressin-induced glucose release by the perfused rat liver.

Livers were perfused as described in the text in the presence of an initial perfusate glucose concentration of 4.33 mM. Vasopressin was added to the medium after 40 mins. perfusion and the rise in perfusate glucose concentration was followed for the next 40 mins. Points are means ± S.E.M. of at least 3 adrenalectomized (●) or sham-operated (○) rats, and 4 adrenalectomized rats injected subcutaneously with cortisol (10 mg.kg^{-1}) four hrs. prior to perfusion (■).

3.2.3 The Effects of Adrenal Steroids and Vasopressin upon Gluconeogenesis in the Perfused Livers of 48 h. Fasted Rats.

In order to investigate whether adrenocorticosteroids could influence hormone stimulated gluconeogenesis as well as glycogenolysis, livers from 48 h. fasted rats were perfused in the presence of 8 mM lactate and 2 mM pyruvate. Such livers released glucose into the perfusion medium at a relatively constant rate and single dose of vasopressin (400 $\mu U.ml^{-1}$) increased this rate by about 50% (figure 18).

The dose dependence curve for vasopressin enhanced glucose release into the perfusate of livers from 48 h. fasted rats is similar for both adrenalectomized and sham-operated control animals (figure 19). Vasopressin was effective in stimulating gluconeogenesis at doses as low as 30 $\mu U.ml^{-1}$, and the maximally effective dose of the hormone was between 200 and 400 $\mu U.ml^{-1}$. Thus, unlike vasopressin induced glycogenolysis, the gluconeogenic potency of the hormone in rats was undiminished by adrenal ablation.

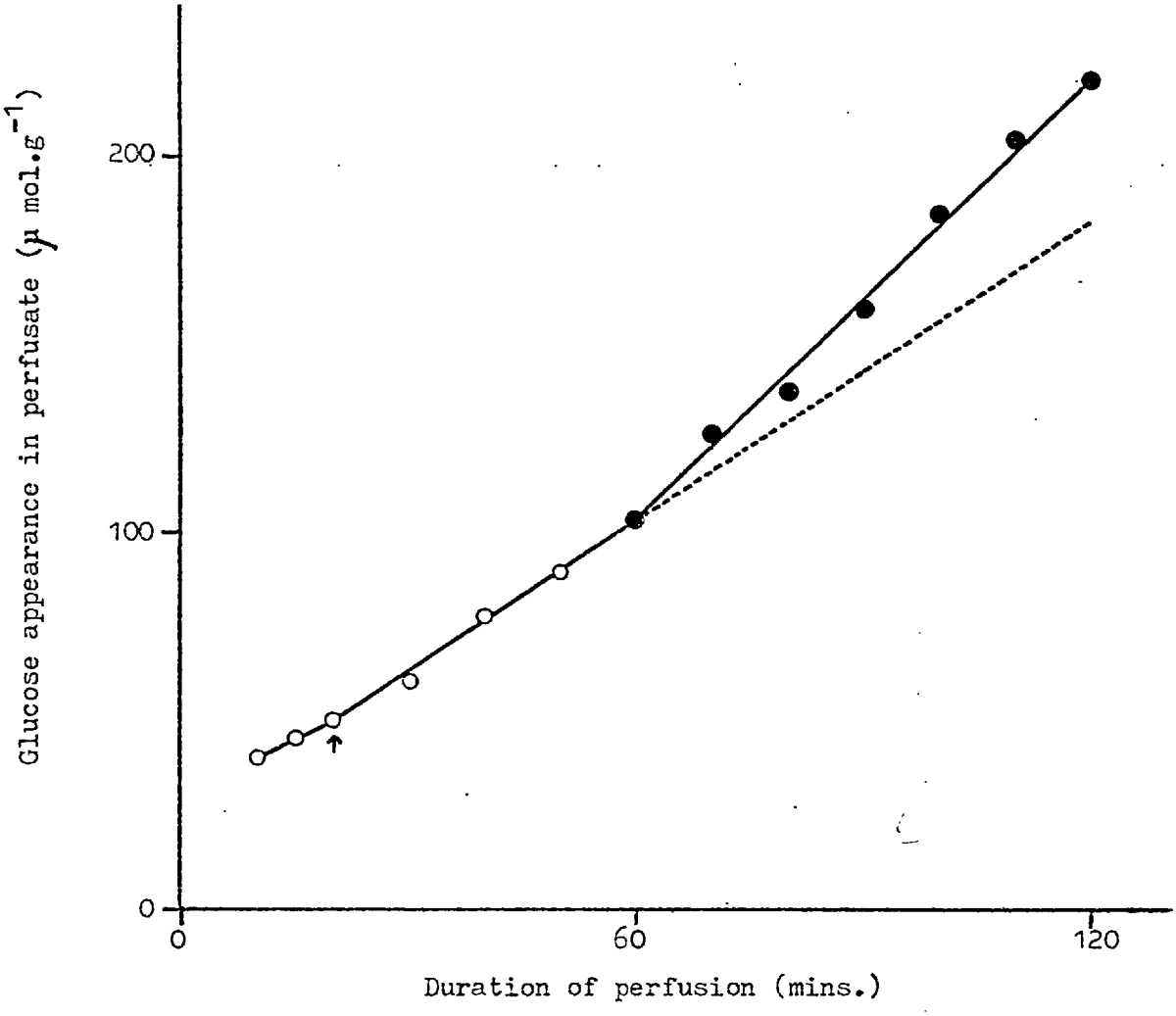


Fig. 18 The effect of vasopressin upon gluconeogenesis in the perfused liver of a 48 h. fasted rat.

The liver of a 48 h. fasted rat weighing 180 gms. before diet restriction was perfused as described in the text(○).Lactate and pyruvate were added to the perfusion medium at 20 mins. (↑) to the initial concentrations of 8 and 2 mM respectively; thereafter, these substrates were infused to maintain these concentrations. Vasopressin (400 μU.ml^{-1}) was added to the perfusate at 60 mins. (●). The results are taken from a single perfusion.

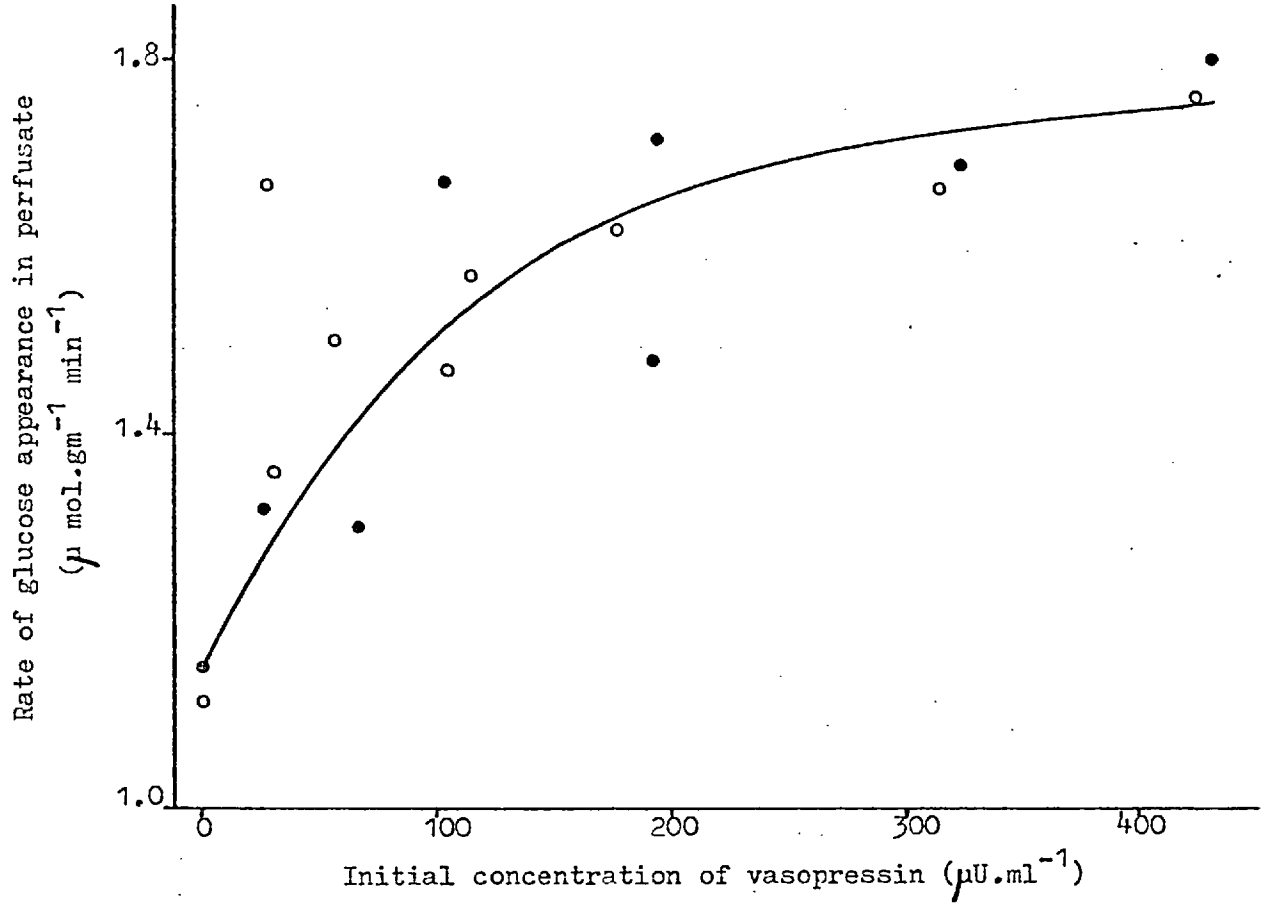


Fig. 19 The effect of vasopressin upon glucose production by the perfused livers of 48 h. fasted rats.

Livers from 48h. fasted rats were perfused as described in the text. Lactate and pyruvate were added at 15 mins. to the initial concentrations of 8 mM and 2 mM respectively, and infused to maintain these concentrations. Vasopressin was added in a single dose at 30 mins. and glucose appearance in the perfusate was measured over the subsequent 30 mins. (●) denotes perfusions from adrenalectomised rats, (o) denotes perfusions from sham-operated control animals.

3.3 Further Studies Concerning the Metabolic Actions of Arginine Vasopressin.

In the previous section, the experiments were designed to clarify the influence of the adrenal gland on the actions of vasopressin upon hepatic carbohydrate metabolism. It is clear that vasopressin is able to exert a direct effect upon the liver since the hormone influences hepatic carbohydrate metabolism when added in vitro to a perfused liver preparation. This direct action of vasopressin is in contrast to the effects of cortisol in adrenalectomized rats since, with regard to fat metabolism at least, cortisol was only able to restore hepatic lipogenesis in adrenalectomized rats if the animals were treated with the hormone in vivo. In view of the interest of these previously unreported direct effects of vasopressin upon hepatic metabolism, experiments were designed to further elucidate the hepatic actions of the hormone, and to shed some light upon the mechanism by which the hormone exerts its metabolic effects.

3.3.1 The Interaction between Vasopressin and Insulin in the Control of Hepatic Glycogenolysis.

Having demonstrated that adrenal ablation exerts a sparing effect upon vasopressin induced degradation of hepatic glycogen (figure 17), experiments were designed to check if insulin could exert a similar effect.

Figure 20 shows the effect of added insulin (5 $mU.ml^{-1}$) upon

the release of glucose into the perfusate caused by a single dose of vasopressin (400 $\mu U.ml^{-1}$). 40 mins. after the addition of vasopressin, the amount of glucose released by the perfused livers in the presence of insulin was about 35% of that released in the perfusions without insulin. Thus, insulin appears to antagonize vasopressin-induced glycogen breakdown in the perfused liver.

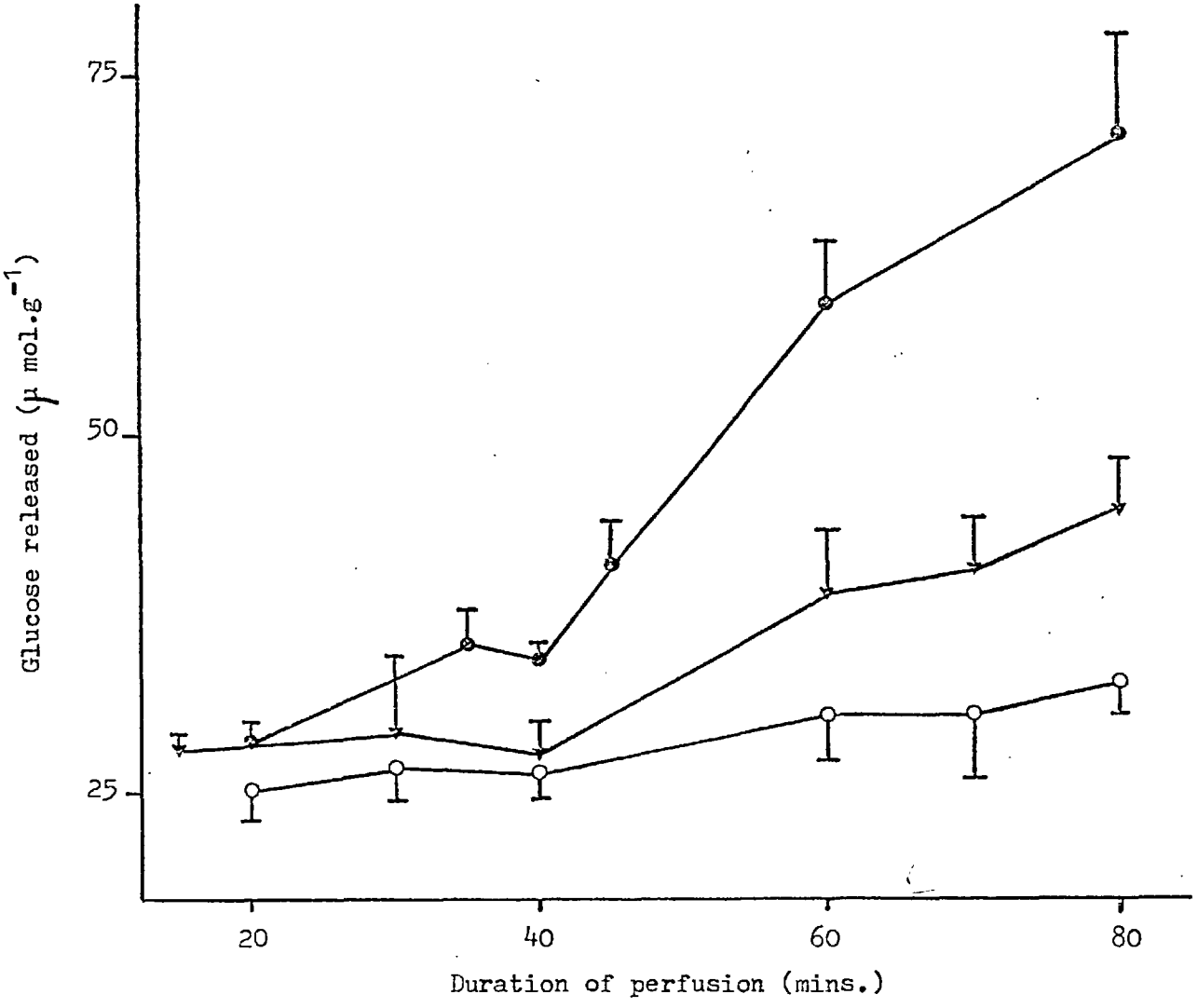


Fig. 20 The interaction between vasopressin and insulin in the control of glucose release by the perfused liver.

Livers from fed rats were perfused as described in the text. After 40 mins. vasopressin was added to an initial concentration of 400 μU.ml^{-1}. Glucose was assayed in samples of perfusion medium removed from the collecting vessel, thus the glucose released during perfusion was calculated. Results are means ± S.E.M. of 3 control perfusions (O), 3 with vasopressin (●) and 4 perfusions with vasopressin to which 5 mU.ml^{-1} insulin were added at 15 min. intervals throughout the perfusion (▼).

3.3.2 The Influence of Vasopressin upon the Concentrations of Hepatic Metabolites.

In figures 21 and 22 is shown the effect of vasopressin upon the tissue concentrations of various metabolic intermediates in the perfused livers of 48 h. fasted and fed rats respectively. The livers from fasted rats were perfused in the presence of 15 mM lactate and 1.5 mM pyruvate in the perfusion medium, whilst those from fed rats were perfused in the presence of 8 - 10 mM glucose alone. In each figure a) illustrates the changes produced by vasopressin in the tissue concentrations of various glycolytic intermediates, while b) illustrates the concomitant alterations in the tissue concentrations of various intermediates associated with the entry of tricarboxylic acid cycle metabolites into the gluconeogenic pathway.

The tissue concentrations of glycolytic intermediates between glucose and the triose phosphates were all greater in fed rats than they were in fasted animals. (Compare control values in legends to figures 21 and 22). The increased levels of these intermediates in the fed state presumably reflect enhanced glucose production in the fed animal mainly resulting from glycogenolysis.

In many respects, the qualitative effects of vasopressin upon the tissue concentrations of glycolytic intermediates are similar in the fed and fasted states (figures 21a and 22a). In the presence of unchanged lactate, the tissue level of pyruvate is markedly diminished, while that of phosphoenol pyruvate is markedly increased. The measured concentrations of metabolites between phosphoenol pyruvate and glucose were increased by vasopressin

treatment with the exception of glucose-6-phosphate in the livers of starved rats which was markedly reduced in concentration by the hormone.

The effects of vasopressin upon the levels of the intermediates shown in figures 21b) and 22b) are also similar to each other in many respects. Thus, in both the fed and fasted states, treatment of the perfused liver with vasopressin resulted in a marked reduction in the tissue concentrations of α-ketoglutarate in the presence of unchanged citrate.levels and increased concentrations of aspartate and phosphoenol pyruvate. The tissue concentration of malate was not significantly altered by vasopressin in the perfused livers of 48 h. fasted rats (where large amounts of lactate were infused into the perfusate). However, in the fed state the concentration of this metabolite was elevated following hormone treatment.

In table 12 is shown the influence of vasopressin upon the tissue concentrations of adenine nucleotides and various intermediates involved in "redox" reactions within the cell. The addition of vasopressin to the perfusion medium of livers from fed rats had no effect upon the hepatic concentration of the three adenosine phosphates measured (AMP, ADP and ATP).

The tissue concentrations of redox metabolites shown in table 12 have been used to calculate the ratios of oxidised to reduced free pyridine nucleotides shown in table 13 (Williamson et. al., 1967; Veech et. al., 1969). The calculations involved rely on the following principal assumptions upon which the estimates for the equilibrium constants of the reactions involved are based: 1) the pH of the mitochondria and cytosol

Fig. 21 The effect of vasopressin upon the concentrations of intermediates in the perfused livers of 48 h. fasted rats.

Fig. 21 The effect of vasopressin upon the concentrations of intermediates in the perfused livers of 48 h. fasted rats.

Livers from 48 h. fasted rats were perfused in the presence of exogenous lactate (15 mM) and pyruvate (1.5 mM) which were infused to maintain a constant concentration. The livers were freeze clamped in situ 10 mins. after the addition of vasopressin (●) (10 $mU.ml^{-1}$) to the perfusate, or 50 mins. after the start of control perfusions to which no hormone was added (O). Intermediates were assayed as described in the text. Results are expressed as mean percentages (± S.E.M.) of the mean control values (± S.E.M.) and are taken from at least 3 perfusions in each group.

Abbreviations used, with the actual control values in parentheses (n mol. g fresh liver $^{-1}$), are as follows : Lact - lactate (14880 ± 3090), Pyr - pyruvate (659 ± 114), PEP - phosphoenol pyruvate (267 ± 40), 2PG - 2 phosphoglycerate (45 ± 12), 3PG - 3 phosphoglycerate (290 ± 36), Tri-P - triose phosphates (15 ± 0.1), FDP - fructose diphosphate (13 ± 4), G6P - glucose-6-phosphate (61 ± 11), Gluc - glucose (3207 ± 577), ∝-kg -∝-ketoglutarate (376 ± 77), Cit - citrate (212 ± 36), Asp - aspartate (501 ± 66).

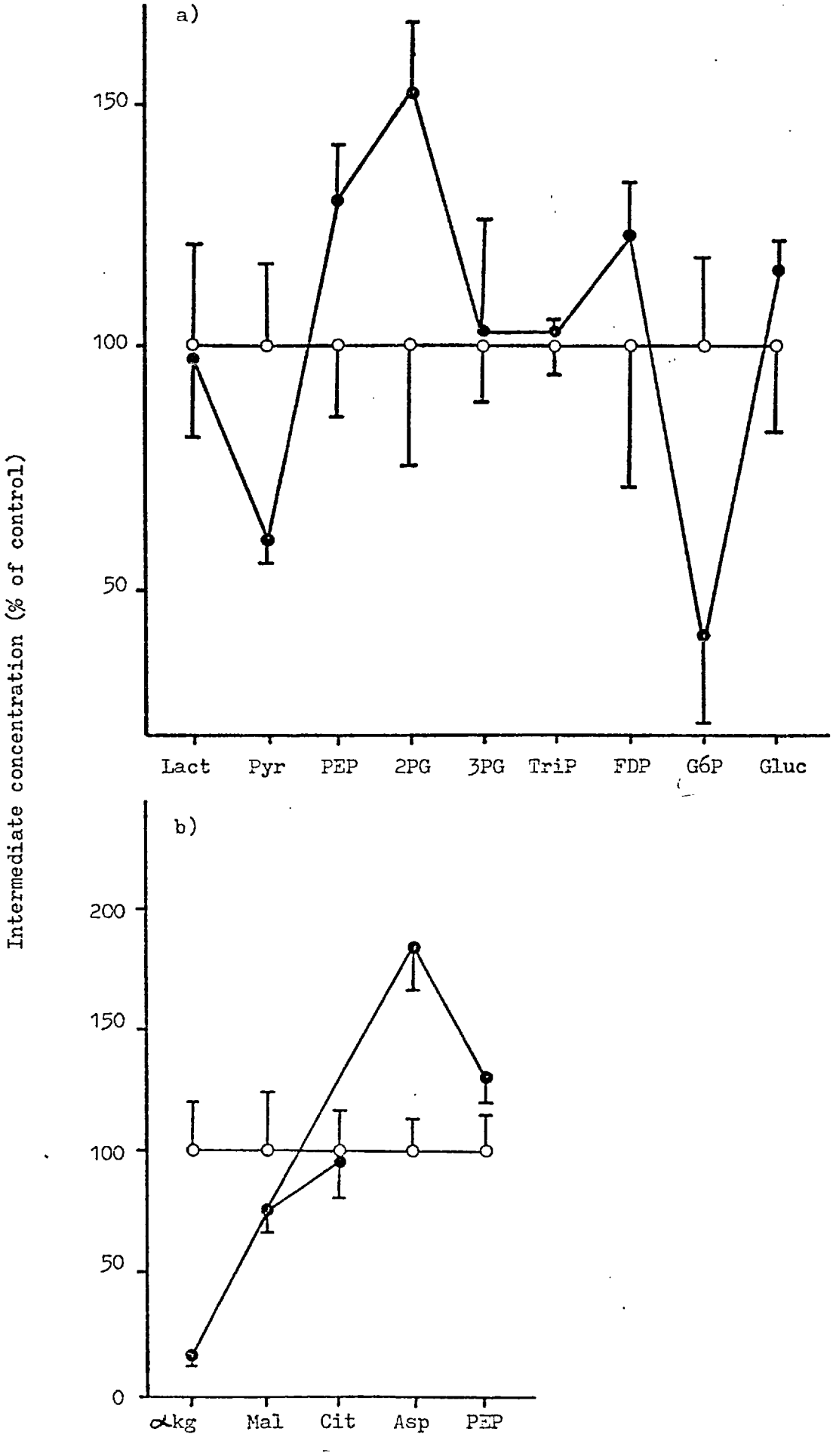
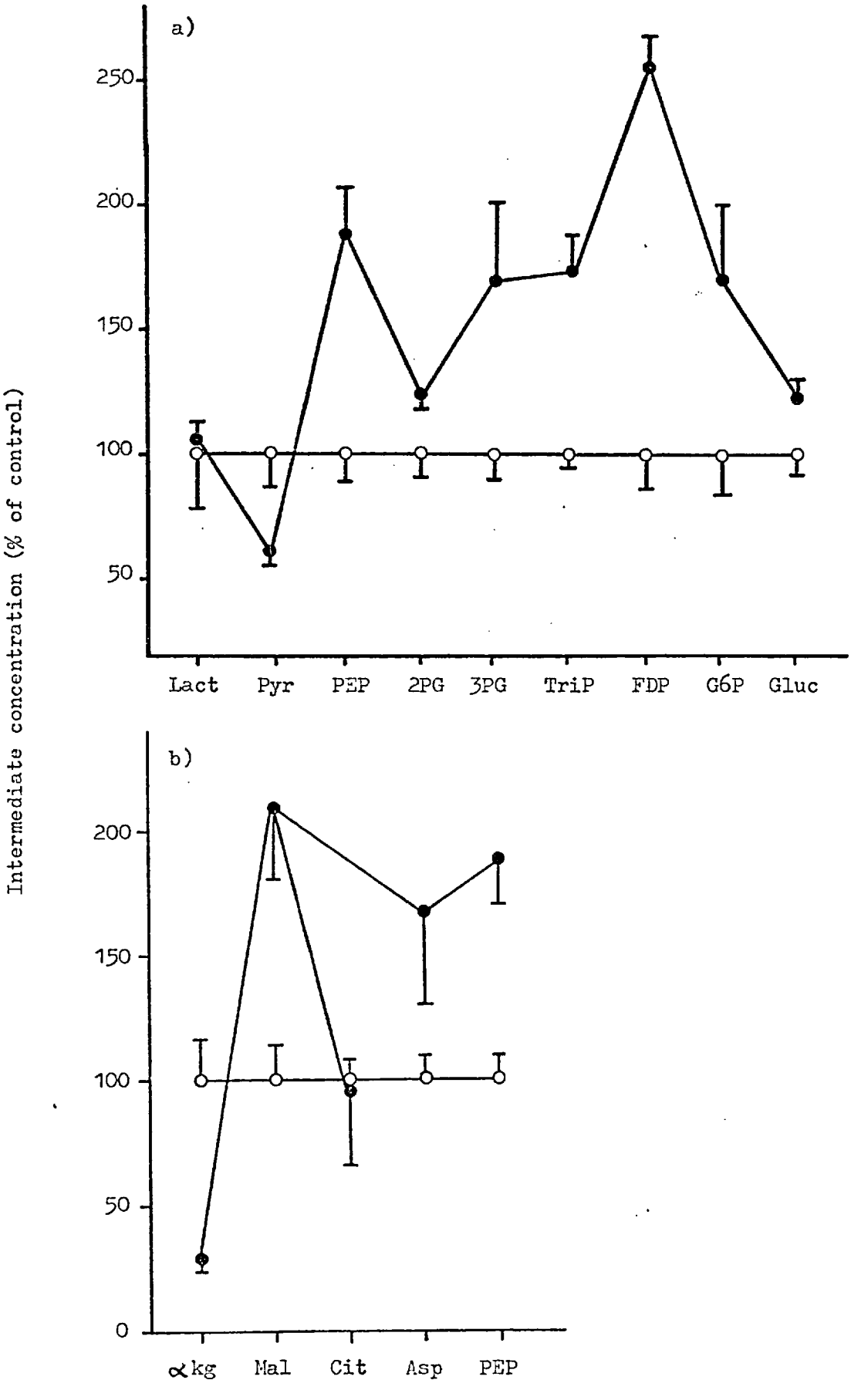

a)
150
100
50
Lact
Pyr
PEP
2PG
3PG
TriP
FDP
G6P
Gluc
b)
200
150
100
50
0
αkg
Mal
Cit
Asp
PEP
Intermediate concentration (% of control)

Fig. 22 The effect of vasopressin upon the concentrations of intermediates in the perfused livers of fed rats.

Fig. 22 The effect of vasopressin upon the concentrations of intermediates in the perfused livers of fed rats.

Livers perfused in the presence of 8 - 10 mM glucose were freeze clamped in situ 10 mins. after the addition of vasopressin (●) (10 $mU.ml^{-1}$) to the perfusate, or 50 mins. after the start of control perfusions to which no hormone was added (O). Intermediates were assayed as described in the text. Results are expressed as mean percentages (± S.E.M.) of the mean control values (± S.E.M.) and are taken from at least 3 perfusions in each group.

Abbreviations used, with the actual control values in parentheses (n mol. g fresh liver $^{-1}$), are as follows : Lact - lactate (2924 ± 637), Pyr - pyruvate (215 ± 29), PEP - phosphoenol pyruvate (37 ± 4), 2 PG - 2 phosphoglycerate (17 ± 2), 3 PG - 3 phosphoglycerate (107 ± 11), Tri-P - triose phosphates (38 ± 2), FDP - fructose diphosphate (25 ± 4), G6P - glucose-6-phosphate (179 ± 30), Gluc - glucose (12070 ± 960), α-kg - α-ketoglutarate (366 ± 61), Cit - citrate (280 ± 21), Asp - aspartate (389 ± 41).


a)
250
200
150
100
50
Lact
Pyr
PEP
2PG
3PG
TriP
FDP
G6P
Gluc
b)
200
150
100
50
0
αkg
Mal
Cit
Asp
PEP
Intermediate concentration (% of control)

is 7.0, 2) the ionic strength of the mitochondria and cytosol is 0.25. The calculations involved also assume that the concentration of the relevant substrates is constant throughout the tissue.

The principal effect of vasopressin upon the redox state of free hepatic pyridine nucleotides appears to involve a shift of the cytoplasmic $[NADP^+]$ / $[NADH]$ ratio to a more reduced state, and a similar change in the cytoplasmic $[NAD^+]$ / $[NADH]$ ratio. These changes were most marked in the fed state. In the starved state there was also a small reduction in the mitochondrial $[NADP^+]$ / $[NADPH]$ ratio.

Having calculated the cytoplasmic free $[NAD^+]$ / $[NADH]$ ratio as described above, and knowing the tissue concentration of malate, the tissue concentration of oxaloacetate has been calculated by assuming the equilibrium constant for malate dehydrogenase (Williamson et. al., 1967). The results of this calculation for both control and vasopressin-treated livers from fed and 48 h. starved rats are shown in table 14. In these experiments, vasopressin treatment resulted in a near halving of the calculated oxalacetate concentration of the perfused livers of starved rats, but exerted no effect upon the calculated concentration of this metabolite in the perfused livers of fed rats. These results will be discussed in relation to the metabolite profiles shown in figures 21 and 22 in the next chapter.

Table 12 The effect of vasopressin upon the concentrations of various redox metabolites and nucleotides in the perfused livers of fed and 48 h. starved rats.

Table 12 The effect of vasopressin upon the concentration of various redox metabolites and nucleotides in the perfused livers of fed and 48 h. starved rats.

Livers, perfused as described in figures 19 and 20, were rapidly frozen in liquid nitrogen 10 min. after the addition of vasopressin (10 $mU.ml^{-1}$) to the perfusion medium, or after 50 min. perfusion in the case of control experiments. Metabolites were measured enzymatically as described in the text. Results are expressed as means $\pm$ S.E.M. of the measurements from at least three separate perfusions. Differences between hormone treated and control groups in the same nutritional state are significant as indicated (* $p < 0.02$).

Metabolite	Concentration in liver (n mol. g fresh liver^{-1})			
	Fed control	Fed + vasopressin	Starved control	Starved + vasopressin
Lactate	2924 ± 637	3003 ± 340	14880 ± 3090	14510 ± 2420
Pyruvate	215 ± 29	133 ± 6	659 ± 114	399 ± 31
Malate	340 ± 46*	712 ± 100*	1294 ± 330	972 ± 123
α-Ketoglutarate	366 ± 61*	107 ± 16*	376 ± 77*	65 ± 4*
Citrate	280 ± 21	267 ± 43	212 ± 36	210 ± 42
β-Hydroxybutyrate	320 ± 47	404 ± 61	222 ± 62	253 ± 30
Acetoacetate	146 ± 42	178 ± 35	60 ± 50	47 ± 19
ATP	2451 ± 218	2563 ± 85	—	—
ADP	940 ± 72	1137 ± 79	—	—
AMP	188 ± 24	181 ± 31	—	—

Table 13 The effect of vasopressin upon the calculated ratios of oxidised to reduced free pyridine nucleotides in the perfused rat liver.

The ratios [free oxidised nucleotide] / [free reduced nucleotide] have been calculated from the results in table 11 according to the methods of Williamson et.al.,(1967) and Veech et.al.,(1969). The concentration of CO_2 in the liver was presumed to be 1 mM, and that of isocitrate equal to 0.056 X that of citrate (Veech et.al.,1969). The relevant equilibrium constants are given in the references cited above.

Pyridine nucleotide ratio	Metabolite ratio employed for calculation	Calculated ratio: Fed control	Fed + vasopressin	Starved control	Starved + vasopressin
Cytoplasmic $[NAD^+]/[NADH]$	[Pyruvate]/[lactate]	662	399	399	248
Mitochondrial $[NAD^+]/[NADH]$	[Acetoacetate]/[β-hydroxybutyrate]	9.25	8.94	5.48	3.77
Cytoplasmic $[NADP^+]/[NADPH]$	[Pyruvate] $[CO_2]$/[malate]	0.018	0.005	0.014	0.012
	[α-Ketoglularate] $[CO_2]$/[isocitrate]	0.020	0.006	0.027	0.005

Table 14 The effect of vasopressin upon the calculated cytoplasmic concentration of oxalacetate in the perfused livers of fed and 48 h. starved rats.

The concentration of oxalacetate in the experiments described in table 12 has been calculated from the formula :

$$[\text{oxalacetate}] = [\text{malate}] \times [NAD^+] / [NADH] \times K$$

where K is the equilibrium constant of malate dehydrogenase and is assumed to equal 2.78×10^{-5} (Williamson et. al., 1967). For this purpose, the concentration of malate in the cytoplasm has been assumed to be equal to that measured in the acid extracts of whole liver.

Experimental group	Cytoplasmic $[NAD^+] / [NADH]$ (from table 13)	$[\text{Malate}]$ (n mol. g^{-1}) (from table 12)	Calculated $[\text{Oxaloacetate}]$ (n mol. g^{-1})
Fed control	662	340	6.3
Fed + vasopressin	339	712	6.7
Starved control	339	1294	12.2
Starved + vasopressin	248	972	6.7

3.3.3 The Influence of Vasopressin upon Hepatic Lipid Metabolism.

It is clear that vasopressin may exert profound effects upon hepatic carbohydrate metabolism in the rat, particularly with regard to glycogen breakdown and gluconeogenesis from lactate. Thus, vasopressin influences the metabolic fate of two major precursors of hepatic fatty acids. Vasopressin treatment in the perfused liver also resulted in a reduction of the free cytoplasmic $[NADP^+]$ / $[NADPH]$ ratio. Since the major role of cytoplasmic NADPH is thought to be the provision of reducing equivalents for fatty acid synthesis (Veech et. al., 1969), it was of interest to study the influence of vasopressin upon hepatic lipid metabolism.

With this aim in mind, two approaches were adopted. Firstly, the influence of vasopressin upon the formation and release of ketone bodies from livers perfused in the presence of circulating sodium oleate (0.025M, pH 8.5) was studied (figure 23). In these experiments, the addition of vasopressin (1 $mU.ml^{-1}$) to the perfusate failed to result in any change in the rate of acetoactetate or β-hydroxybutyrate release from the perfused liver.

In another set of experiments, the total rate of hepatic fatty acid synthesis and release, and the proportion of those fatty acids derived from lactate as a carbon source was measured using ^3H_2O and (^{14}C)-lactate (table 15). These experiments were performed with a total perfusate volume of 100 mls. in order to minimise the rise in circulating glucose concentration which occurred when vasopressin was added to the perfusion medium. Also, in contrast to previous experiments with (^{14}C)-lactate, a mixture of

(^{14}C)-lactate : pyruvate (8:1), of the same specific activity as that initially added, was infused into the perfusion medium for the hour over which fatty acid synthesis was measured. In this way a more valid measurement of the proportion of newly synthesized fatty acids derived from (^{14}C)-lactate could be made. In these experiments CO_2 was trapped in NaOH as it left the oxygenator as described in section 2.4.5, and thus the conversion of circulating lactate into CO_2 could be calculated.

The results shown in table 15 fail to demonstrate any significant effect of vasopressin upon the synthesis and release of hepatic fatty acids, or the contribution of lactate carbon to the synthesis of these fatty acids. Similarly, there is no significant effect of vasopressin upon the conversion of lactate to CO_2.

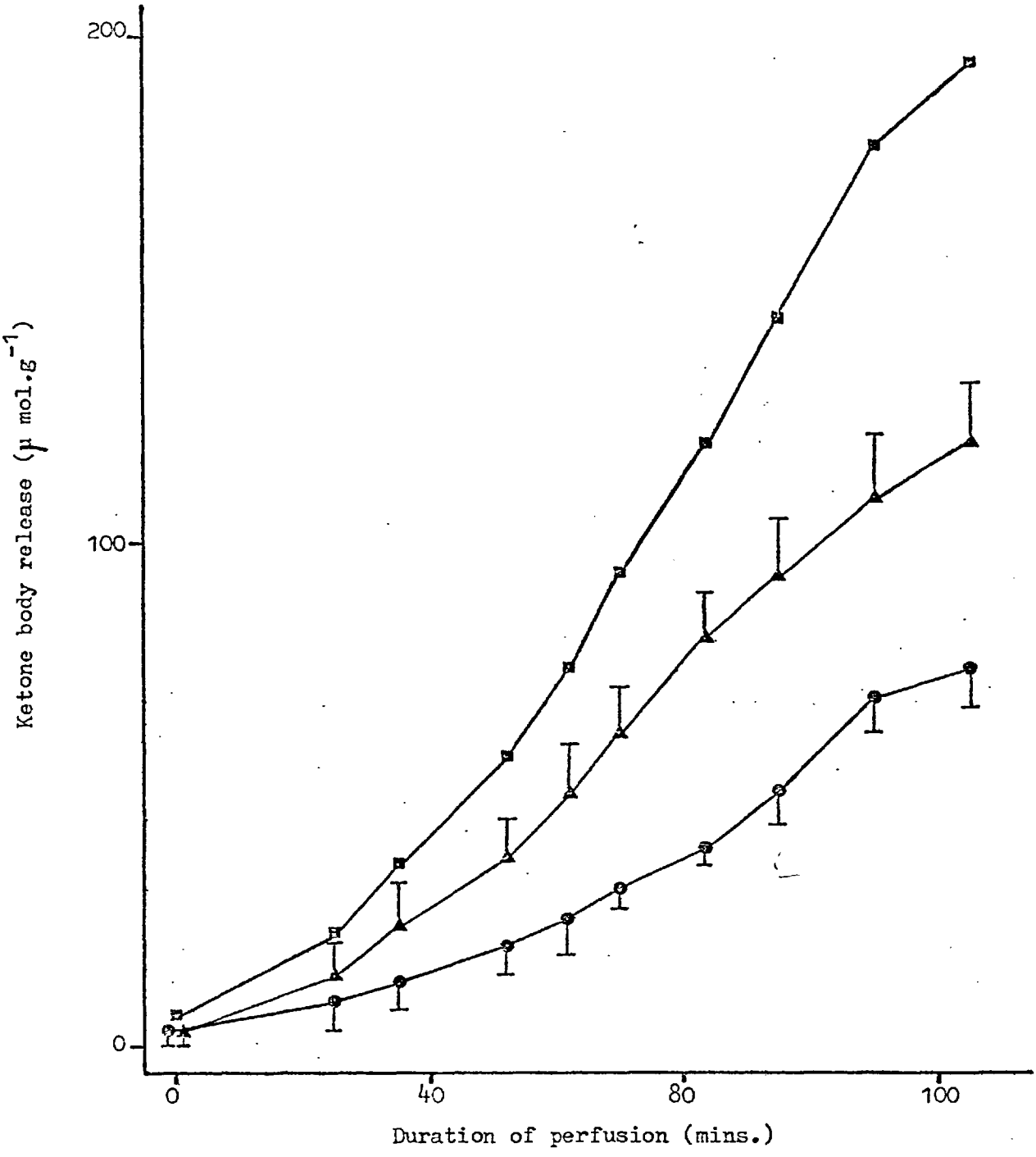


Fig. 23 The influence of vasopressin upon the hepatic release of ketone bodies into perfusion medium.

Livers from 48 hr. fasted rats weighing 180 gms. prior to starvation were perfused as described in the text. Sodium oleate (0.025M, pH 8.5) was infused into the perfusate at the rate of 3 ml.h^{-1} and vasopressin 1 mU.ml^{-1} was added at 70 mins. The concentration of acetoacetate (●) and β-hydroxybutyrate (▲) in de-proteinised perfusate samples was measured as described in the text. ■ denotes the total release of these two ketones from the perfused liver. Results are means $\pm$ S.E.M. of the measurements from 3 perfusions.

TABLE 15 The influence of vasopressin upon fatty acid synthesis, and the conversion of lactate into CO_2 in the perfused livers of fed rats.

Livers of fed rats were perfused in the month of April as described in the text, the total perfusate volume was 100 ml. ^3H_2O and (^{14}C)-lactate: pyruvate (8:1) were added at 40 mins. (^{14}C)-lactate: pyruvate (8:1) of the same specific radioactivity was infused into the perfusion medium for the next hour (over which fatty acid synthesis was measured), in order to maintain a constant concentration of the radioactive precursor. Circulating glucose and lactate concentrations remained approximately constant at 15 and 16 mM respectively throughout the determination of fatty acid synthesis. CO_2 released from the perfusate was trapped as described in the text, and thus the conversion of lactate to CO_2 could be calculated. Results are means ± S.E.M. of the number of observations indicated. Differences between the control and vasopressin-treated groups are not significant for any parameter.

Experimental group	No. of observations.	Fatty acid synthesis (umol C_2 units.g^{-1}.h^{-1}.)				CO_2 derived from lactate (μ gm.atoms.g^{-1}.h^{-1}.)
		Total		% from lactate		
		Liver	Medium	Liver	Medium	
Control	4	19.1±2.8	4.2±1.2	52.5±6.6	68.7±13.3	79.8 ± 4.2
Vasopressin treated	3	15.5±3.6	2.7±0.6	64.6±6.9	52.8± 5.6	62.8 ± 7.2

3.3.4 The Influence of Vasopressin and other Hormones upon Hepatic Concentrations of Adenosine -3', 5'- cyclic Monophosphate.

In recent years much attention has been directed towards the mechanism of action of various hormones. Many hormones appear to exert some of their short term effects by influencing the intracellular concentration of adenosine -3, 5- cyclic monophosphate (Robison et. al., 1971). It is clear that vasopressin exerts short term effects upon hepatic metabolism, hence it was of interest to study the possible role of cyclic AMP in these effects.

In intact anaesthetized rats, glucagon and adrenalin injected into the hepatic portal vein increased the hepatic concentration of cyclic AMP in the expected fashion (figure 24). The elevated levels of hepatic cyclic AMP caused by these two hormones were of the same order as those previously reported (Exton et. al. (1971a, 1972b), Vasopressin (10 mU or 100 mU) administered in the same fashion led to no detectable increase in the hepatic concentration of cyclic AMP.

It has been suggested that the release of cyclic AMP into circulating perfusate is a more sensitive index of hormonal effects upon hepatic adenyl cyclase than the hepatic concentration of the nucleotide (Exton and Park, 1972). This idea stems from the possibility that pools of the nucleotide exist within the liver which are not susceptible to hormone action. With this idea in mind, the effects of these hormones upon the concentration of cyclic AMP in the effluent medium from perfused livers was investigated.

In the perfused liver, glucagon and adrenalin caused the

expected increase (figure 25) in the concentration of cyclic AMP in the effluent medium (Exton et. al. 1971a, 1972b). However, vasopressin (10 $mU.ml^{-1}$ and 100 $mU.ml^{-1}$) failed to increase effluent cyclic AMP concentration, despite causing glucose output which was of the same order as that previously observed (see figure 16), and that produced by adrenalin (figure 26).

It has been reported that the activity of the adrenalin - sensitive hepatic adenyl cyclase is increased following adrenalectomy (Bitensky et. al., 1970), and that basal and hormone stimulated cyclic AMP levels are increased in fasted rats perfused in the presence of theophylline (Exton et. al., 1971b). In view of these findings, cyclic AMP was assayed in the effluent perfusate from the liver of a 48 h. starved, adrenalectomized rat perfused in the presence of 1 mM theophylline (figure 25). While the basal effluent cyclic AMP concentration was elevated three fold in this experiment, release of the nucleotide was not increased in response to vasopressin (100 $mU.ml^{-1}$).

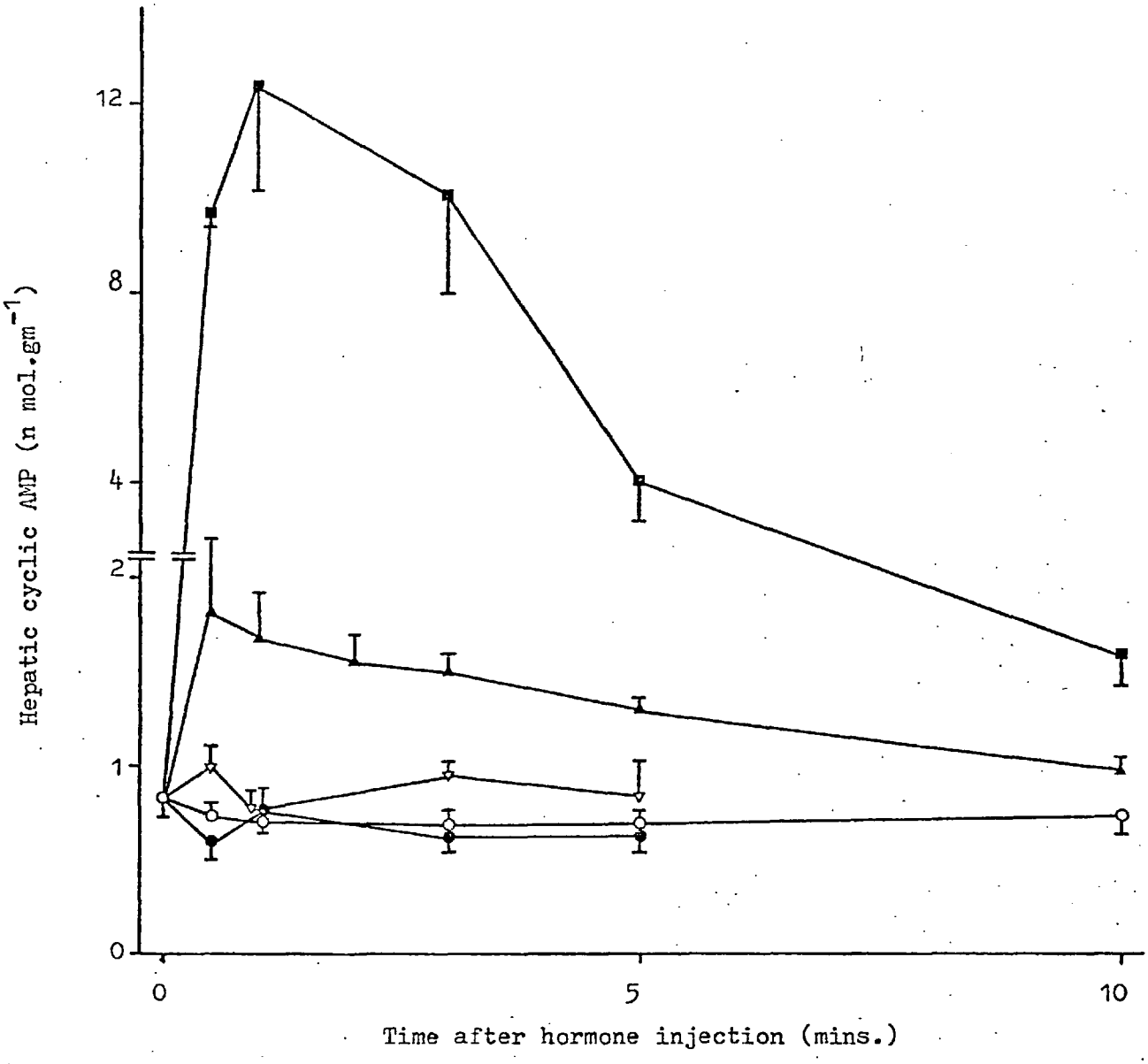


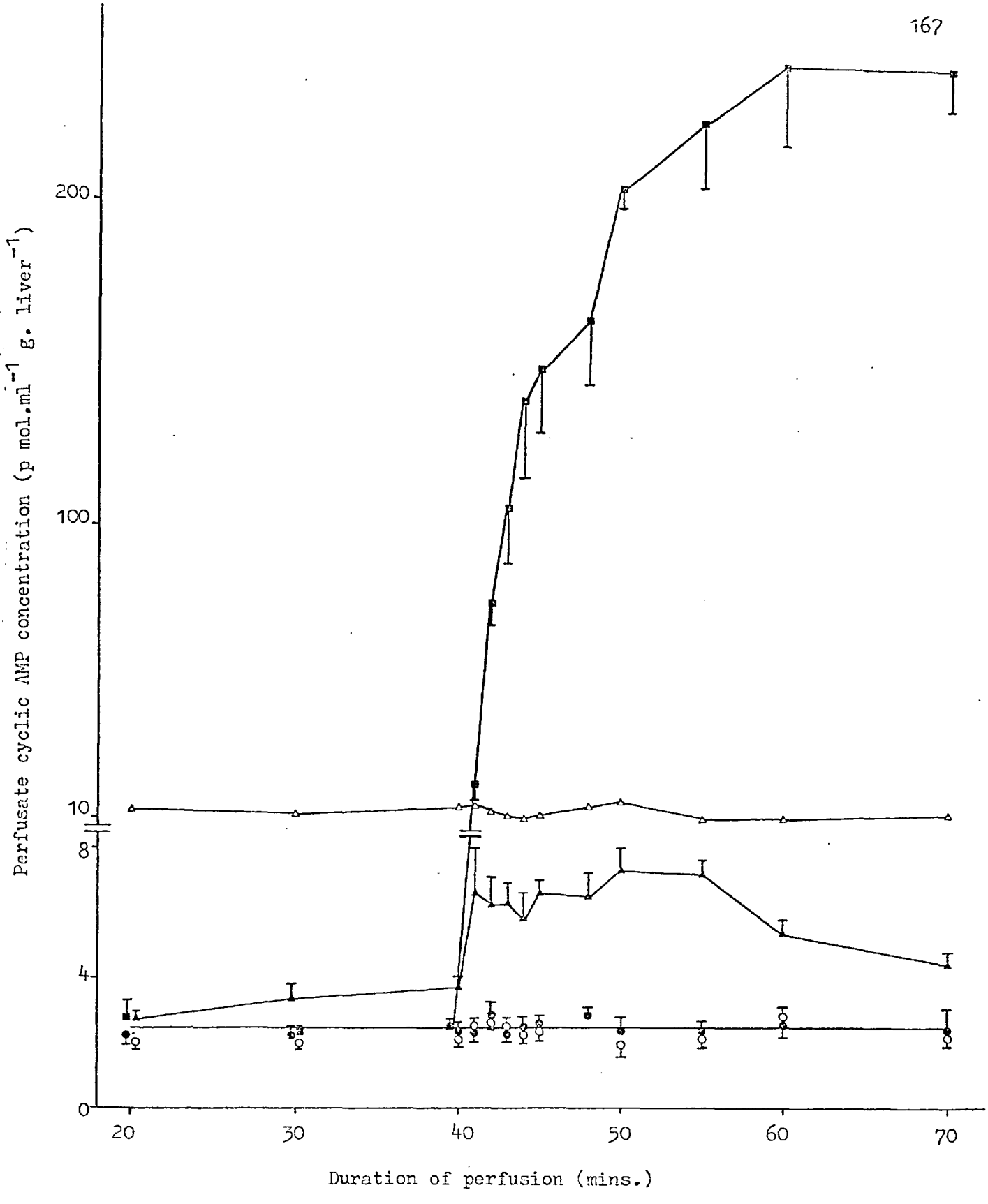
Fig. 24 The effect of hormones upon hepatic cyclic AMP in vivo.

Fed rats were anaesthetised and the hepatic content of cyclic AMP was determined at various times after injection into the hepatic portal vein of : glucagon, 1 μgm (■); adrenalin, 1.5 x 10^{-8} mol (▲); vasopressin 10 mU (●) or 100 mU (▽); or 0.9% NaCl (○). Other details are in the text. Results are means ± S.E.M. of 3 - 6 measurements.

Fig. 25 The effect of hormones upon effluent perfusate cyclic AMP from the perfused liver.

Fig. 25 The effect of hormones upon effluent perfusate cyclic AMP from the perfused liver.

Livers were perfused as described in the text, and cyclic AMP was measured in the dripping effluent medium collected at the times indicated in the figure. Hormones were added after 40 mins. perfusion to the following initial concentrations : glucagon, 10 $ng.ml^{-1}$ (■); adrenalin, $10^{-6}M$ (▲); vasopressin, 100 $mU.ml^{-1}$ (●), no additions (○). Results are means ± S.E.M. from 3 perfusions. Also shown in the figure is the effect of vasopressin (100 mU/ml) upon effluent cyclic AMP from the liver of a 48 h. fasted adrenalectomized rat perfused in the presence of 1 mM theophylline (△).


Perfusate cyclic AMP concentration (p mol.ml^{-1} g. liver^{-1})
200
100
10
8
4
0
20
30
40
50
60
70
Duration of perfusion (mins.)

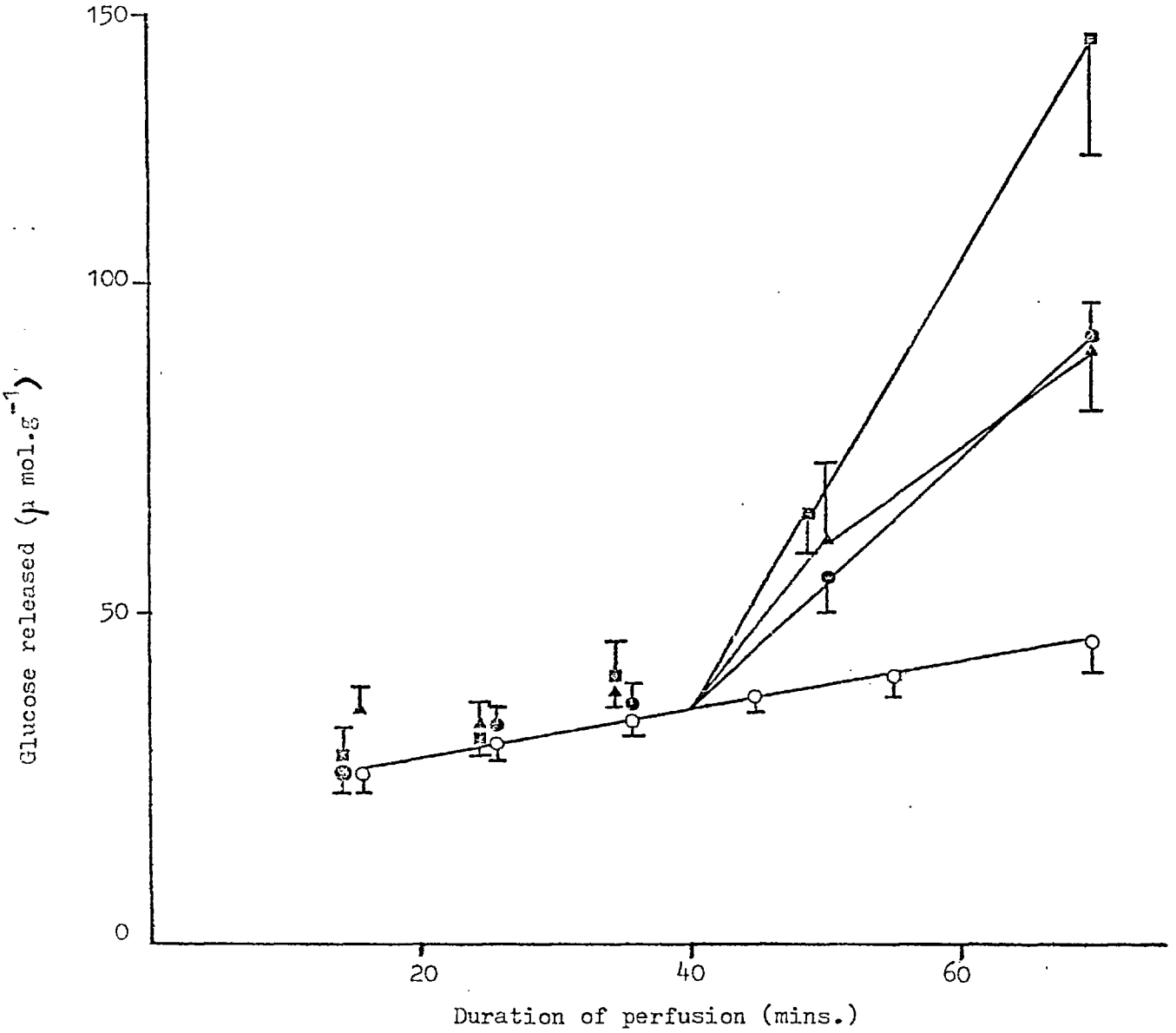


Fig. 26 The effect of hormones upon hepatic glucose output.

In the experiments described in fig. 25 perfusate glucose was measured in a sample of perfusion medium removed from the collecting vessel. The initial glucose concentration at the start of the perfusion was 4.3 mM, and the extra glucose released during perfusion was calculated. Hormones were added at 40 mins. as described in fig. 25 (glucagon ■ , adrenalin ▲ , vasopressin ● , no additions O). Results are means ± S.E.M. from 3 perfusions.

3.3.5 The Influence of Glycogenolytic Hormones upon the Activity of Glycogen Phosphorylase in Intact Rats.

In the experiments described in figure 24, samples of liver were rapidly frozen in liquid nitrogen for the subsequent determination of glycogen phosphorylase (E.C.2.4.1.1.). The enzyme assays were performed by Dr. P. Whitton (Department of Biochemistry, Imperial College, London). The results of this study have been published (Hems et. al., 1975b), and these results are shown in figure 27 with the kind permission of Dr. P. Whitton.

It is clear from the results shown in figure 27 that a single injection of vasopressin (10 m units) into the hepatic portal vein resulted in a short-term activation of glycogen phosphorylase. This activation of the enzyme, which was similar to that observed following adrenalin injection (1.5×10^{-8} mol.), reached a maximum value within 30 secs. of hormone injection. A larger dose of vasopressin (100 m units) resulted in an activation of glycogen phosphorylase which was similar in magnitude to that produced by the smaller dose, but which was sustained for a longer period of time. In contrast, glucagon injection (1 μg) resulted in a much greater activation of glycogen phosphorylase, which only reached a maximum value 3 mins. after the injection of the hormone.

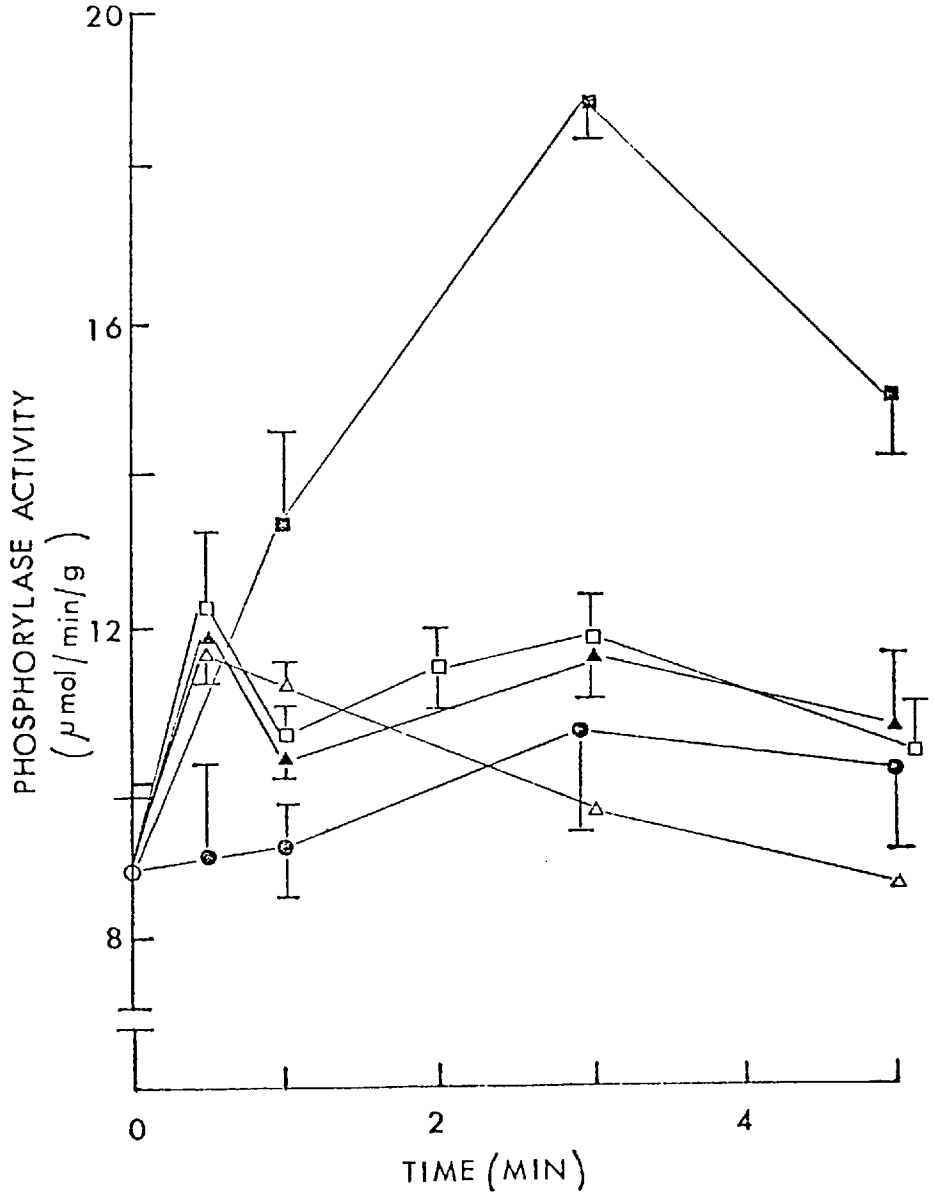


Fig. 27 The time course of glycogen phosphorylase activation by glycogenolytic hormones in vivo.

In the experiments described in fig. 24, glycogen phosphorylase activity was measured in samples of rapidly frozen liver. The hormones were injected into the hepatic portal vein in the following quantities : glucagon, 1 ug (■), adrenalin, 1.5 x 10^{-6} Mol (□), vasopressin, 10 mU (▲), 100 mU (△). (●) represents control injections of 0.9% NaCl (0.25 ml). Results are means ± S.E.M. of at least 3 measurements, except for vasopressin (100 mU) at 3 and 5 mins., where the results are the mean of 2 measurements.

3.3.6 The Influence of Vasopressin and other Hormones upon Hepatic Concentrations of Guanosine -3',5'-cyclic Monophosphate.

In view of the suggestion that guanosine -3, 5- cyclic monophosphate may have a 'second messenger' role in hormone action similar to that of cyclic AMP, the hepatic concentration of cyclic GMP, and that in effluent perfusate, was measured in response to several hormones (table 16). Of the four hormones tested (glucagon, adrenalin, vasopressin and insulin) only glucagon exerted an effect on cyclic GMP (in the effluent medium) which might be regarded as significant ($p < 0.05$). All the other hormones failed to influence the concentration of cyclic GMP in the perfusate, and no hormone affected the hepatic concentration of the nucleotide. The putative effect of glucagon upon the release of cyclic GMP by the perfused liver was very small by comparison with the effects of this hormone upon cyclic AMP.

Table 16 The effect of various hormones upon the accumulation and release of guanosine -3 , 5- cyclic monophosphate in the perfused liver.

Fed male rats weighing 180gms were perfused as described in the text in the presence of an initial concentration of 5 mM glucose in the perfusion medium. Hormones were added to the perfusion medium so as to achieve the concentrations indicated after an initial perfusion period of 40 mins. After a further 10 mins of perfusion effluent medium was collected as described in the text, and the liver was freeze clamped in situ prior to cyclic GMP determination as previously described. Results are expressed as means $\pm$ S.E.M. of the number of observations indicated. There are no significant differences between groups with regard to either the hepatic or circulating concentrations of cyclic GMP.

Experimental group	Hormone addition	No. of observations	Concentration of cyclic GMP: Liver (pmol g^{-1})	Effluent perfusate (pmol ml^{-1})
1	None	3	14 $\pm$ 4	0.8 $\pm$ 0.1
2	Glucagon ($10\ mg.ml^{-1}$)	3	13 $\pm$ 3	1.4 $\pm$ 0.3
3	Adrenalin ($10^{-5}M$)	3	24 $\pm$ 3	1.3 $\pm$ 0.4
4	Vasopressin ($100\ mU.ml^{-1}$)	3	17 $\pm$ 3	0.8 $\pm$ 0.3
5	Insulin ($5\ mU.ml^{-1}$)	3	17 $\pm$ 2	0.8 $\pm$ 0.3

3.3.7 The Role of Extracellular Ca^{++} in the Metabolic Actions of Vasopressin.

It is clear from the results described in the last section that vasopressin does not exert its metabolic effects by increasing the hepatic concentration of cyclic AMP. Hence, it was of interest to consider an alternative mechanism. One possible such mechanism could involve cation-dependent events as have been implicated in the hormonal control of hepatic gluconeogenesis (Tolbert and Fain, 1974; Pilkis et. al., 1975).

During the course of a study of the calcium-dependence of vasopressin-induced glucose release, it emerged that glucose release from the perfused liver was critically temperature sensitive. Thus, experiments were designed to test both the calcium ion dependence and the temperature sensitivity of this hormonal effect. The results of these experiments are plotted as regression lines of hepatic glucose release versus perfusate temperature in figure 28. A comparison of the slope of lines from perfusions with normal Ca^{++} concentration (solid) with that of the lines from perfusions with no added Ca^{++} in the medium (dotted) demonstrates that the temperature sensitivity of vasopressin-induced glucose release is greater in the absence of exogenous Ca^{++}. Similarly, the fact that the lines for perfusions with vasopressin (solid symbols) are parallel to those with no hormone (open symbols) for each concentration of circulating Ca^{++}, indicates that the temperature sensitivity of hepatic glucose output is the same whether or not vasopressin is present.

It is clear from figure 28 that changes in temperature result

in glucose output (or uptake) in the perfused liver. However, since the two regression lines (with and without hormone) are parallel for each concentration of exogenous Ca^{++}, it is clear that the hormone stimulated glucose release from the perfused liver is not temperature sensitive. For each concentration of circulating Ca^{++}, the vertical distance between the two parallel regression lines represents the hepatic glucose release attributable to vasopressin. Clearly, in the absence of added Ca^{++}, the magnitude of the vasopressin-stimulated release of hepatic glucose is reduced to about 50% of that observed in the presence of normal circulating Ca^{++} levels.

Fig. 28 The effects of Ca^{++} concentration and temperature upon basal and vasopressin-stimulated glucose release by the perfused liver.

Fig. 28 The effects of Ca^{++} concentration and temperature upon basal and vasopressin-stimulated glucose release by the perfused rat liver.

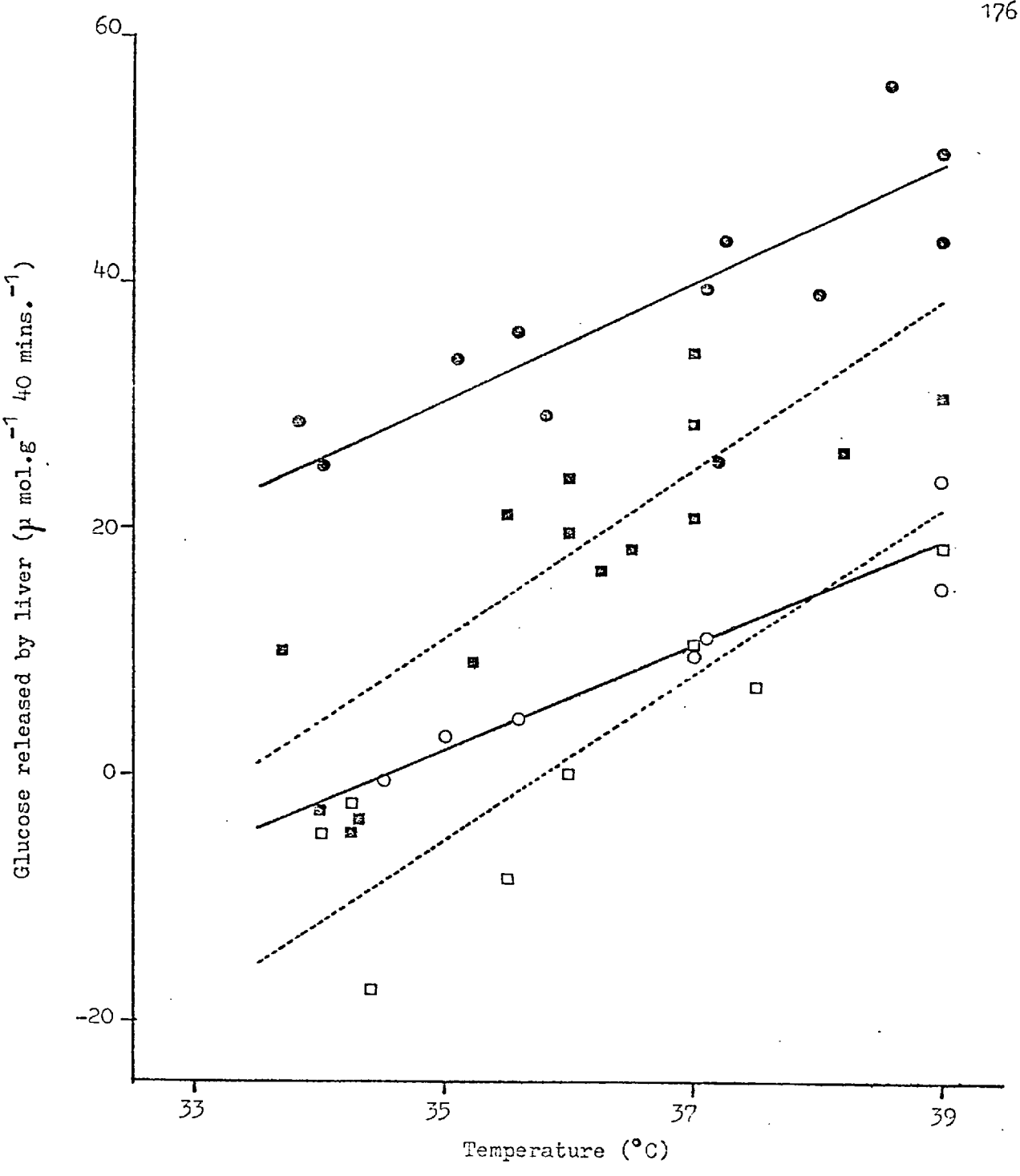
Livers were perfused as described in the text, with glucose added to the initial concentration of 4.3 mM. The temperature of the perfusion cabinet was thermostatically controlled so that the temperature of the perfusion medium entering the liver was maintained between 33°C and 39°C (± 0.5°C). Regression lines were plotted for livers perfused with buffer containing Ca^{++} at the usual concentration (2.52 mM.) (circles and solid lines), and those perfused with Ca^{++} free buffer (squares and dotted lines). Glucose release was measured between 40 and 80 mins. of perfusion, and in some perfusions vasopressin (500 $\mu U.ml^{-1}$) was present during this period. The equations for the regression lines and their correlation coefficients (r), where x = temperature and y = hepatic glucose release, are as follows :

2.52 mM. exogenous Ca^{++}, no vasopressin $y = 4.31x - 149$ ($r = 0.95$) ○

500 $\mu U.ml^{-1}$ vasopressin $y = 4.76x - 136$ ($r = 0.79$) ●

No exogenous Ca^{++}, no vasopressin $y = 6.79x - 243$ ($r = 0.90$) □

500 $\mu U.ml^{-1}$ vasopressin $y = 6.88x - 229$ ($r = 0.88$) ■


60
40
20
0
-20
33
35
37
39
Temperature (°C)
Glucose released by liver (μ mol.g^{-1} 40 mins.$^{-1}$)

4 Discussion.

4.3.2 The Influence of Insulin upon Vasopressin-induced Glucose Release from the Perfused Liver.

4.3.3 The lack of a Role for Cyclic Nucleotides in the Hepatic Actions of Vasopressin.

4.3.4 The Role of Extracellular Ca^{++} Ions in Vasopressin Action upon the Liver.

4.3.5 A Summary of the Information Currently Available Concerning the Mechanism by which Vasopressin Influences Hepatic Metabolism.

4 Discussion

4.1 The Control of Lipogenesis by Adrenal Corticosteroids in the Rat

4.1.1 The Influence of the Adrenal Gland upon the de novo Synthesis of Fatty Acids in the Perfused Liver.

The results shown in table 4 indicate that the perfused livers of adrenalectomized rats exhibit a consistently reduced capacity for fatty acid synthesis as compared to sham-operated controls. These results were obtained in conditions which are optimal for hepatic fatty acid synthesis (Salmon et.al.,1974). The results are in agreement with the findings of Perry and Bowen (1955,1956) who reported that the incorporation of (^{14}C)-acetate into fatty acids in liver slices was reduced following adrenalectomy in the rat.

The existence of seasonal variations in hepatic lipid metabolism has been clear for some time (Thorp,1963), although this aspect of lipid metabolism has not been extensively studied. In the present study, the rate of hepatic fatty acid synthesis was greatest during the winter months (Nov–Jan), and smallest in the summer (July,Sept.). This pattern of seasonal variation is not the same as that reported for circulating lipids by Thorp (1963), who observed two annual peaks in serum lipids, in the spring and autumn. However, it is possible that other seasonal peaks of fatty acid synthesis would emerge if the process was studied at more frequent regular intervals throughout the year.

Following adrenalectomy, the impairment of total fatty acid synthesis was associated with a decline in the rate of

export of newly synthesised fatty acids from the liver, as indicated by the appearance of such fatty acids in the perfusion medium (table 4). Analysis of lipid extracts from perfusion medium indicated that 75% of the radioactivity associated with newly synthesised fatty acids was located in triacylglycerols and this proportion was not altered following adrenalectomy (results not shown). The above results are thus in agreement with the suggestion of Heimberg (1966) that the release of triacylglycerol by the liver is proportional to the amount of fatty acid available to the tissue. These results are also compatible with the results of Klausner and Heimberg (1967) who showed that net triacylglycerol release (not merely that of newly synthesised fatty acids) from the perfused rat liver was diminished following adrenalectomy and restored to normal by cortisone treatment in vitro. The involvement of adrenocorticoid hormones in the control of hepatic triglyceride release is further indicated by the work of Reaven et.al.(1974), who demonstrated that corticosteroids induce hyperlipoproteinaemia by enhancing the production of hepatic VLDL.

The reduced rate of synthesis and export of fatty acids in the perfused livers of adrenalectomized rats was associated with a 50% decline in the contribution of monoenoic fatty acids to the total rate of de novo synthesis (table 5). This diminution in monoenoate synthesis, and it's subsequent restoration following cortisol treatment in vivo, are in keeping with the frequently observed parallelism between the synthesis of monoenoates and total fatty acids. Such parallelism has also been observed in

starved and diabetic animals, and as a function of age (Gellhorn and Benjamin, 1966; Mercuri et.al., 1974).

In livers perfused under the present conditions, fatty acids are synthesised mainly from glycogen, lactate and to a lesser extent, glucose (Salmon et.al., 1974). An attempt was made to assess whether there was any specific impairment in lipogenesis from these precursors in the livers of adrenalectomized rats. The initial hepatic glycogen levels of the adrenalectomized rats used in these experiments (table 4) did not significantly differ from those of the sham-operated controls; thus there was no apprent shortage of glycogen in the adrenalectomized group.

The contribution of glucose or lactate carbon to total fatty acid synthesis was similar in both experimental groups. Furthermore, it is clear from figure 14, that under the present conditions the perfused livers of adrenalectomized and sham-operated rats maintain similar concentrations of lactate and glucose in the perfusion medium. Thus the reduced capacity for fatty acid synthesis exhibited by the perfused livers of adrenalectomized rats does not reflect a specific impairment in the incorporation of any single precursor into hepatic fatty acids.

The activities of various enzymes associated with fatty acid synthesis have been reported to be diminished following adrenal ablation in the rat. Thus Lardy et.al.,(1965) have shown that the activity of hepatic ATP citrate lyase was reduced following adrenalectomy, and restored to normal after five days treatment with hydrocortisone. The Diamants and their coworkers

(Diamant and Shafrir, I975; Diamant et. al., I975) have shown that the synthetic steroids triamcinolone and dexamethasone, when administered in vivo, produce a rapid rise in the hepatic activity of acetyl CoA carboxylase and a slower increase in fatty acid synthetase activity. These observations are compatable with the results of the present study. In contrast, Volpe and Marasa (I975) reported that the hepatic activities of acetyl CoA carboxylase and fatty acid synthetase were not altered following either adrenalectomy or hydrocortisone treatment in vivo. Thus it seems that the synthetic steroids used by the Diamants may not resemble hydrocortisone in their action upon hepatic fatty acid synthesis. However, the results of Volpe and Marasa (I975) are not strictly at odds with the findings of the present study which indicated that while hepatic fatty acid synthesis was diminished by adrenal ablation in the perfusion conditions described here, the process was unaffected in vivo.

Several workers have reported that adrenalectomy may reverse the effects of diabetes upon hepatic lipogenesis in the rat (Linder and Migliorini, I974; Volpe and Marasa, I975). Such reports appear to be inconsistent with the results of the experiments described in table 4. In the present study adrenalectomy, performed five days after the induction of streptozotocin diabetes and five days before liver perfusion, failed to increase the diminished rate of fatty acid synthesis measured during liver perfusion. Volpe and Marasa (I975) found

that the reduced hepatic activities of acetyl CoA carboxylase and fatty acid synthetase in intact diabetic rats were increased following adrenalectomy. This observation supported the earlier work of Linder and Migliorini (1974), who reported that the incorporation of (^{14}C)-acetate into the hepatic fatty acids of diabetic rats in vivo was enhanced following adrenal ablation. However, in the same study, Linder and Migliorini (1974) demonstrated that adrenalectomy failed to influence fatty acid synthesis from (^{14}C)-acetate in liver slices prepared from diabetic rats. Thus the effect of adrenal ablation upon the reduced rates of hepatic fatty acid synthesis observed in diabetic rats does not appear to be demonstrable in vitro. Furthermore, in the studies described above, adrenalectomy was performed at the same time or before the induction of diabetes, whereas in the present study the adrenal glands were removed only when the animals exhibited definite symptoms of diabetes (elevated blood glucose and loss of weight). When comparing their results with those of earlier workers, Linder and Migliorini (1974) concluded that the effects of adrenal excision upon diabetic animals are only fully manifested if the glands are removed before the metabolic changes induced by diabetes are well established. If this is the case, then the failure of adrenalectomy to influence hepatic fatty acid synthesis in the diabetic animals used in the present study is not surprising. Furthermore, if the precise timing of the induction of adrenal insufficiency can influence the qualitative effects of such insufficiency upon the metabolism of the diabetic rat, then the physiological significance of these effects is difficult to assess. However, the general

implication of this observation is that adrenal glucocorticoids might have a role in the control of enzyme degradation rather than enzyme synthesis. It is possible that the apparent restoration of hepatic fatty acid synthesis resulting from the early adrenalectomy of a diabetic rat could reflect a more complex sequence of events during insulin deficiency rather than a direct hepatic response to lack of either insulin or corticosteroids (e.g. adrenalectomy-induced changes in other hormone levels).

4.1.2 The Adrenal Glucocorticoid Control of Fatty Acid Synthesis in Intact Rats.

Experiments in vivo failed to demonstrate the decreased capacity for fatty acid synthesis observed in the perfused livers of adrenalectomized rats. Hepatic fatty acid synthesis in both fed rats, and those pretreated with intra-gastric glucose, was not influenced by adrenal ablation (table 3). These results are in accord with a previous study of Fain and Wilhelmi (1962) who used $^{3}H_2O$ to measure hepatic lipogenesis. The present results are also in keeping with the failure of exogenous cortisol to influence hepatic lipogenesis in vivo (Volpe and Marasa, 1975). As discussed in section 1.2.2, studies concerning the role of the adrenal gland in hepatic fatty acid synthesis in vivo have produced inconsistent results. This inconsistency, and the failure of the present study to reveal a diminution in hepatic fatty acid synthesis in the intact

adrenalectomized rat, may be explained by the relatively small extent of the intrinsic change following adrenalectomy (revealed here in the controlled conditions of liver perfusion), and by the fact that this impairment in fatty acid synthesis is seasonally variable. Also, many previous studies concerning fatty acid synthesis in vivo have used ^{14}C-labelled precursors which can lead to underestimates of total rates of hepatic fatty acid synthesis (Hems et.al., 1975a).

In the present study, while adrenalectomy failed to influence hepatic fatty acid synthesis in vivo, the accumulation of de novo synthesised fatty acids in epididymal adipose tissue was diminished in intact adrenalectomised rats pretreated with intra-gastric glucose (table 3). This result is in contrast to other reports which suggest that glucocorticoids reduce, and adrenal ablation increases the activity of the enzymes acetyl CoA carboxylase and fatty acid synthetase in the adipose tissue of intact rats (Diamant and Shafrir, 1975; Volpe and Marasa, 1975). Similarly, reports that the incorporation of ^{14}C-labelled precursors into adipose tissue fatty acids in vitro is reduced 3.5h. after cortisol administration (Munck and Koritz, 1960), and unaffected by adrenalectomy (Jeanrenaud and Renold, 1960) are not consistent with the results of the present study.

De Gasquet et.al., (1975) have recently shown that the activity of adipose tissue lipoprotein lipase in both fed and fasted intact rats was increased by glucocorticoid injection and diminished following adrenalectomy. Thus it is possible that the reduced rate of accumulation of de novo synthesised fatty acids in adipose tissue is a result of an impaired uptake of

newly synthesised fatty acids released as VLDL from the liver.

In the present experiments with intact rats, the relative contribution of glucose to hepatic fatty acid synthesis as compared to that in adipose tissue fatty acid synthesis was inferred from the quotient:

$$\frac{^{14}C \text{ d.p.m.}}{^{3}H \text{ d.p.m. in adipose tissue fatty acids}} \Big/ \frac{^{14}C \text{ d.p.m.}}{^{3}H \text{ d.p.m. in liver fatty acids.}}$$

This quotient was about 3-5 in all experimental groups, thus confirming the relative unimportance of glucose as a precursor for hepatic fatty acid synthesis (Salmon et.al., 1974); the quotient was not influenced by adrenalectomy.

4.1.3 The Influence of Adrenal Glucocorticoids upon Hepatic Cholesterogenesis in the Perfused Liver.

A study of the de novo synthesis of cholesterol in some of the experiments described in table 4, did not reveal a consistently discernible effect of adrenal ablation upon this process in the perfused rat liver (table 8). However, in the experimental group which had exhibited the greatest inhibition of fatty acid synthesis following adrenalectomy (group 1a) there was a significant elevation of cholesterogenesis. This observation is in agreement with the work of Hickman et.al., (1972) who reported that the circadian rhythm of cholesterogenesis was abolished following adrenalectomy in the rat, the resultant rate of synthesis remaining uniformly elevated during the 24h cycle.

In another experimental group (perfusions performed in April) the elevation of cholesterol synthesis following adrenalectomy was not statistically significant. Similarly, the induction of streptozotocin diabetes did not influence total cholesterogenesis in the subsequently perfused liver (table 8), but the contribution of glucose carbon to the total rate of synthesis was markedly reduced. This observation of unchanged hepatic cholesterogenesis after adrenalectomy is in keeping with the results of Huber et.al.,(1972) who showed that the circadian rhythm of hepatic β-hydroxy β-methylglutaryl CoA reductase activity was not affected by adrenal ablation in the rat. These results were supported by the work of Nervi and Dietschy (1974) who demonstrated that the incorporation of (^{14}C)-octanoate into cholesterol was unaffected by adrenalectomy when measured in liver slices prepared from rats whose nutritional status had been carefully controlled. The results of Huber et.al. (described above)are not in agreement with the earlier work of Edwards (1973) who reported that hepatic β-hydroxy β-methylglutaryl CoA reductase activity was diminished following the abolition of its normal circadian rhythm as a result of adrenal insufficiency.

Thus, despite considerable precautions to control such variables as lighting and nutritional state, no consensus exists with regard to the influence of the adrenal gland upon hepatic cholesterogenesis. This situation is not clarified by the present study in which the qualitative nature of the effect of adrenalectomy upon hepatic cholesterol synthesis was found to vary during the year. It is possible that this seasonal variation in cholesterogenesis is partly responsible for the confusion which surrounds the question of

the adrenocortical control of the process.

4.1.4 The Role of the Adrenal Gland in the Control of Newly Synthesised Fatty Acid Incorporation into Hepatic Glycerides

The separation of hepatic lipid classes from the perfused livers of adrenalectomised rats revealed a more marked diminution in the incorporation of newly synthesised fatty acids into triacylglycerols than into phospholipids (table 5). This observation which is indicative of a reduced rate of hepatic triacylglycerol synthesis may account for the diminished release from the perfused liver of newly synthesised fatty acids as VLDL which was discussed previously.

It thus appears that the perfused liver of the adrenalectomized rat has a specific impairment in the ability to esterify newly synthesised fatty acid to triacylglycerol. This finding is in agreement with the work of Hays and Hill (1965) who demonstrated that the ability of rat liver microsomes to incorporate (^{14}C)-palmitate into triacylglycerol was enhanced following 7 days cortisol treatment in vivo. The influence of the adrenal gland upon the synthesis and release of hepatic triacylglycerols may be relevant to the elevation of these processes in obesity. Hems and his coworkers (Salmon and Hems, 1973; Hems et.al., 1975a) have reported enhanced synthesis and export of hepatic triacylglycerols in genetically obese mice, which exhibit hyperfunction of the adrenal cortex (Herberg and Kley, 1975).

The question arises of whether the diminished incorporation of newly synthesised fatty acids into the hepatic glycerides of adrenalectomised rats is a direct result of the reduced level of fatty acid synthesis in such livers. Henly et.al.,(1963)have reported that hepatic levels of α-glycerol phosphate are diminished following adrenal ablation in the rat. It might be suggested that the diminution in hepatic triacylglycerol synthesis in the adrenalectomized rat is a consequence of the reduced availability of α-glycerol phosphate for esterification. However, it is not likely that the hepatic concentration of this metabolite is ever limiting to triacylglycerol synthesis in the liver.

The incorporation of albumin-bound exogenous fatty acids into hepatic glycerides occurred at the same rate in the perfused livers of both adrenalectomized and sham operated rats. Thus, given an adequate supply of fatty acid, the perfused liver of the adrenalectomized rat is able to synthesise triacylglycerols and phospholipids at the normal rate. The reduced rate of triacylglycerol synthesis exhibited by the perfused livers of adrenalectomized rats, appears to reflect a mechanism by which the diminished hepatic pool of newly synthesised fatty acid available to such animals is channelled towards maintaining synthesis of 'structural lipids' (phospholipids) at the expense of triacylglycerol synthesis.

In the perfusions with exogenous oleate, de novo fatty acid synthesis was reduced compared with non-oleate controls and was similar in the perfused livers of both adrenalectomized and sham-operated rats. However, this was not the case in the perfusions with circulating palmitate. Thus, while the results of the experiments with exogenous oleate might be taken to suggest that

the impairment of fatty acid synthesis following adrenalectomy could be due to an excess of hepatic free fatty acid (e.g. from lipolysis) the results of the perfusions with palmitate do not support this hypothesis. The impaired lipogenic capacity in the livers of adrenalectomised rats perfused with circulating palmitate was characterised by a diminished rate of incorporation of newly synthesised fatty acids into both phospholipids and especially triacylglycerols. Hence, the livers of adrenalectomized rats perfused in the presence of added palmitate exhibited the same disproportionate inhibition of triacylglycerol fatty acid synthesis as was first noted in the absence of exogenous fatty acid, while those perfused with added oleate did not.

In the rat liver, newly synthesised oleic acid is preferentially incorporated into triacylglycerols to the extent that 47% of the fatty acid found in this lipid class is oleic acid, while the oleate content of hepatic phospholipids is only 9% (Wood and Harlow, 1969). Thus it is possible that the reduced rate of fatty acid incorporation into triacylglycerols following adrenalectomy could be partially due to the reduced availability of newly synthesised hepatic oleate in these animals (table 5). If this was the case then the presence of oleate in the perfusate might be expected to increase the rate of incorporation of endogenous palmitate into the hepatic triacylglycerols of adrenalectomised rats. This follows because palmitate is the major product of hepatic fatty acid synthesis and constitutes 27% of total hepatic triacylglycerol fatty acid (Wood and Harlow, 1969). Hence, the impairment in monoenoate synthesis exhibited by the perfused livers of adrenalectomized rats (table 5) could be at least partially responsible for the diminished incorporation of newly

synthesised fatty acids into hepatic triacylglycerols in these animals.

The well known inhibition of de novo fatty acid synthesis by excess free fatty acid was more marked in the perfusions with palmitate, than it was in those with oleate. The greater inhibition of fatty acid synthesis in the former experiments probably reflects the greater concentration of circulating fatty acid (0.95mM palmitate c.f. 0.7mM oleate in the latter perfusions). However, the activities of ATP-citrate lyase, fatty acid synthetase and acetyl CoA carboxylase in vivo have been reported to be marginally more inhibited by methyl palmitate than by methyl oleate when the esters were administered orally (Muto and Gibson, 1970). Hence, the lower rate of de novo fatty acid synthesis in the presence of palmitate could reflect a more marked inhibition of fatty acid synthesis by the saturated fatty acid itself.

4.1.5 The Restoration of Hepatic Lipogenesis in the Adrenalectomized Rat by Glucocorticoid Treatment in vivo.

The observation that cortisol pretreatment restores the lipogenic capacity of subsequently perfused livers from adrenalectomised rats is in keeping with the notion that their diminished rate of hepatic fatty acid synthesis is a consequence of reduced concentrations of circulating adrenal glucocorticoids.

The restoration of normal fatty acid synthesis by the injection of these relatively low doses of cortisol ($10mg.kg^{-1}$) is in contrast

to the effect of larger doses of the hormone ($100mg.kg^{-1}$, table 4). The inhibition of hepatic fatty acid synthesis and release by this larger dose of cortisol is in keeping with the work of Brady et.al., (1951), who demonstrated that the injection of cortisone (5-20 mg. day^{-1}) into rats for 3 days inhibited the incorporation of (^{14}C)-acetate into hepatic fatty acids in subsequently prepared liver slices. Thus, not merely the quantitative, but also the qualitative effects of corticosteroids upon hepatic fatty acid synthesis appear to be critically dose-dependent.

In the experiments described in figure 15 there was a peak in hepatic fatty acid synthesis 5.2 h after cortisol injection in vivo. The existence of this peak probably reflects the use of a single dose of the hormone which would be degraded by the perfused liver with a relatively short half-life (Berliner et.al.,1962) While hepatic fatty acid synthesis was maximally restored 5.2 h after cortisol injection in vivo, the release of newly synthesised fatty acids into the perfusion medium was not restored to normal until 50 mins later. This delay could reflect a latent period between the appearance of newly synthesised fatty acids in the perfused liver itself, and the release of these fatty acids into the perfusion medium. The observation that cortisol treatment in vivo can enhance the release of de novo synthesised fatty acids from the perfused livers of adrenalectomized rats is in general agreement with the results of Klausner and Heimberg (1967). These workers demonstrated that large doses of cortisol administered in vivo or in vitro could enhance net triacylglycerol release from the perfused livers of adrenalectomized or normal rats.

Although the hepatic capacity for fatty acid synthesis and release was restored within 6 h of cortisol injection in vivo, there was no such restoration of the relative rate of newly synthesised fatty acid incorporation into triacylglycerols (compared with phospholipids). This is clear from the ratio: dpm in triacylglycerol fatty acid/dpm in phospholipid fatty acid, which was unaffected by cortisol pretreatment. Thus it seems likely that the previously noted specific inhibition of triacylglycerol synthesis in the perfused livers of adrenalectomized rats is not merely the consequence of diminished fatty acid synthesis.

4.1.6 A Possible Role for Insulin in the Effects of Glucocorticoids upon Hepatic Lipogenesis.

The restoration of fatty acid synthesis in the perfused livers of adrenalectomised rats following cortisol treatment in vivo establishes that glucocorticoids can promote fatty acid synthesis when administered to the whole animal. This restoration also indicates that the impaired synthesis of hepatic fatty acids could solely reflect glucocorticoid deficiency. It seems reasonable to assume that corticosterone (which is the major circulating glucocorticoid hormone in rodents) would promote hepatic fatty acid synthesis in a similar fashion to the cortisol used in the present study, since these two steroids generally resemble each other closely in structure and action. However, while cortisol treatment

in vivo restored fatty acid synthesis, desaturation and release from the subsequently perfused livers of adrenalectomized rats, no direct hepatic action of cortisol was apparent when the hormone was added in vitro to perfusions lasting 5.5 h (table 8). Hence, the question arises as to whether glucocorticoids (in vivo) act directly on the liver to influence fatty acid synthesis. Although Klausner and Heimberg (1967) found that high concentrations of cortisol could stimulate net triacylglycerol release from the perfused liver, the experiments described in table 4 showed that large doses of cortisol may not resemble more physiological concentrations in their actions upon fatty acid metabolism. Thus it is possible that glucocorticoids do not normally influence fatty synthesis by direct hepatic action.

The characteristics of the impaired fatty acid synthesis revealed in the present study resemble those which might be expected to accrue from mild insulin lack. Thus, in the experiments described in table 4, the de novo synthesis of hepatic fatty acids was reduced in the perfused livers of diabetic rats. This result confirms many previous studies, including those of Haft and Miller (1956, 1958) who showed that the incorporation of (^{14}C)-acetate into fatty acids was diminished in the perfused livers of alloxan diabetic rats. Further, the impaired lipogenesis in both adrenalectomized and diabetic rats was particularly revealed by a decline in ^{3}H incorporation into triacylglycerols. The release of hepatic triacylglycerol has been shown to be diminished following the induction of diabetes in vivo (Basso and Havel, 1970), and in the perfused liver (Wilcox et.al., 1968). The induction of diabetes has also been shown to impair hepatic synthesis of monoenoic fatty

acids, both in vivo (Mercuri et.al., 1974), and in vitro (Gellhorn and Benjamin, 1966).

While the present results indicate that corticosteroids fail to influence lipogenesis in vitro, this is not the case with insulin. Haft and Miller (1958) reported that the addition of insulin to the perfusion medium restored the rate of (^{14}C)-acetate incorporation into fatty acids in the perfused livers of diabetic rats towards normal, and Haft (1967) reported that insulin added in vitro to normal liver perfusions significantly increased the conversion of (^{14}C)-acetate into fatty acids. Topping and Mayes (1972) have shown that insulin can promote hepatic lipogenesis and triacylglycerol export when added in vitro to liver perfusions. Similarly, recent work by Salmon and Hems (1975) has shown that the addition of insulin to perfusate enhances monoenoate synthesis in the mouse.

Thus the results of the present study are compatible with the hypothesis that the diminution of hepatic lipogenesis following adrenalectomy is the result of mild insulin deficiency. Van Lan et.al.(1974) reported that adrenalectomised rats exhibited reduced levels of plasma insulin compared to normal animals and this result was confirmed in the present study (table 10). Diamant and Shafrir (1975) reported that the glucocorticoid-induced increase in hepatic acetyl CoA carboxylase activity did not occur in alloxan diabetic rats, and suggested that glucocorticoids promote hepatic lipogenesis though hyperinsulinaemia. Promotion of hepatic glycogen synthesis by glucocorticoids in vivo may also involve insulin action (Whitton and Hems 1976).

It follows from the above considerations that other underlying characteristics of the impaired lipogenesis observed in adrenalectomised rats should resemble those in diabetic rats. A study of the literature reveals that this is indeed the case. There have been many measurements of enzyme activities reported for adrenalectomised and diabetic rats. In some enzyme groups (e.g. those concerned with gluconeogenesis) there are qualitative differences between these states. However, reported changes in the hepatic activities of acetyl CoA carboxylase and fatty acid synthetase following adrenalectomy in the rat are negligable (Volpe and Marasa, 1975). No reported data appear to be incompatible with the notion that the diminution of hepatic lipogenesis following adrenalectomy reflects hypoinsulinaemia. Similarly, the corticosteroid-induced increase in the hepatic activities of these enzymes does not occur in alloxan diabetic rats (Diamant and Shafrir, 1975), and hence appears to involve insulin action.

The reported changes in other hepatic enzyme activities following adrenalectomy go someway towards explaining the diminished lipogenesis observed in such animals. Thus, the activity of hepatic citrate cleavage enzyme is diminished in the adrenalectomised rat (Lardy et.al., 1965) and also in diabetic animals (Lowenstein and Kornacker, 1964). Some enzymes of the pentose phosphate pathway have also been shown to be sensitive to adrenal and insulin insufficiency. The hepatic activity of glucose-6-phosphate dehydrogenase has been reported to be diminished in adrenalectomised (Reynolds et.al., 1971) and diabetic rats (Glock and McLean, 1955), although this diminution may be

dependent upon the nutritional state of the animals. In a comprehensive study of the hormonal control of the pentose phosphate pathway, Novello et.al.(1969) demonstrated that the hepatic activity of transketolase was reduced in both adrenalectomised and diabetic rats, and restored to normal following the injection of cortisone and insulin respectively.

In section 4.I.2 it was suggested that the diminished accumulation of newly synthesised fatty acids in the epididymal adipose tissue of adrenalectomised rats could reflect reduced lipoprotein lipase activity (De Gasquet et.al.,1975). This reduced lipoprotein lipase activity could be due to hypoinsulinaemia in adrenalectomised rats, since insulin has been shown to enhance epididymal lipoprotein lipase activity in vitro.(Robinson and Wing, 1970).

Therefore, the results of the present study suggest that the moderate impairment of hepatic fatty acid synthesis in the adrenalectomised rat could reflect an hepatic response to mild insulin lack. Similarly, restoration of hepatic fatty acid synthesis and release by cortisol treatment in vivo could be mediated by insulin, perhaps through direct hepatic action.

4.2 The Control of Hepatic Carbohydrate Metabolism by Adrenal Corticosteroids in the Rat.

4.2.1 The Influence of the Adrenal Gland upon Hepatic Glycogen Synthesis.

It is clear from the results discussed in the last section that adrenal glucocorticoids can exert a stimulatory effect upon hepatic lipogenesis. Since fatty acid synthesis in the liver consumes carbohydrate precursors, these results direct attention towards the influence of adrenal cortical secretions upon hepatic carbohydrate metabolism.

Since the classical observation of Britton and Silvette (1932) that starved adrenalectomized rats are not able to synthesise glycogen at normal rates there have been many studies confirming this general conclusion. The results presented in table 11 demonstrate a reduced capacity for hepatic glycogen synthesis in intact fasted adrenalectomized rats. An intra-gastric dose of glucose, administered 90 mins. before the determination of hepatic glycogen synthesis, partially restored the capacity for this process. These results are in agreement with those of B.Friedmann et.al. (1967) who showed that although the fasted adrenalectomized rat exhibited normal glycogen synthesis within 2 hours of refeeding, maximal levels of liver glycogen were not attained in these animals. This defect was reversed by cortisol treatment for three days.

There have been a number of reports indicating that the activity of hepatic glycogen synthetase a is diminished in

adrenalectomized rats.This diminution is associated with reduced synthetase b phosphatase activity which may be restored by glucocorticoid treatment in vivo (Hornbrook et.al., 1966; Mersmann and Segal, 1969). Nichol and Goldberg (1972) have demonstrated that the early effect of cortisol in inducing glycogen synthetase a activity in the starved adrenalectomized rat was lost during the development of alloxan diabetes. These workers suggested that the early effects of glucocorticoids in promoting glycogen synthesis were the result of steroid induced insulin release. This hypothesis was extended by Miller et.al.,(1973) who reported that glucose infusion into liver perfusions from normal and adrenalectomized rats resulted in an activation of glycogen synthetase and a deactivation of glycogen phosphorylase. This control was absent following the induction of alloxan diabetes. From these results, Miller et.al.,(1973) postulated that insulin and not corticosteroids is an essential prerequisite of hepatic glycogen synthesis. This suggestion is in accord with the results of the present study, since the pretreatment of adrenalectomized rats with intragastric glucose in vivo would be expected to induce insulin release. It seems however, that the impairment of hepatic glycogen synthesis following adrenalectomy is not merely the result of diminished insulin secretion. This follows from the work of Whitton and Hems (1976) who demonstrated that while the activity of glycogen synthetase a and the capacity for net glycogen synthesis in the subsequently perfused livers of adrenalectomized rats were restored following cortisol or insulin treatment in vivo, neither hormone was active

when added in vitro. Thus, while the restoration of hepatic glycogen synthesis in the starved adrenalectomised rat appears to involve insulin release, (as does the restoration of fatty acid synthesis in the fed animal), it seems that this hepatic action of insulin is not independent of other factors. It is possible that insulin and corticosteroids may act synergistically to promote the synthesis of hepatic glycogen.

4.2.2 The Influence of the Adrenal Gland upon Hormone-stimulated Hepatic Glycogen Breakdown.

It was of interest to study the influence of glucocorticoids upon hepatic glycogen breakdown in the fed animal since glycogen is thought to be a major precursor of fatty acids in the liver (Salmon et.al.,1974). Corey and Britton (1941) first demonstrated that an adrenal cortical extract is capable of preventing glycogenolysis in the perfused rat liver. Schaeffer et.al.,(1969) reported that adrenal ablation resulted in reduced hepatic glycogen phosphorylase activity in vivo. These workers also found that adrenalin did not exert it's usual

stimulatory effect upon the activity of this enzyme in adrenalectomized rats. Exton et.al.,(1972a)confirmed the above results, and showed that the glycogenolytic effects of physiological concentrations of glucagon or cyclic AMP were abolished in the perfused livers of adrenalectomized rats. However, high concentrations of the hormones or cyclic AMP did stimulate glycogen breakdown.

In the present work, one particular glycogenolytic state was selected for study. Thus the glucose-mobilising effect of 8-arginine vasopressin (Hems and Whitton, 1973) has been studied in relation to a possible permissive involvement of the adrenal gland. Vasopressin stimulated the release of glucose from the perfused liver at a rate similar to that observed in the presence of adrenalin or glucagon. Furthermore, the circulating levels of the hormone required to stimulate hepatic glucose release are similar to those which may occur in the blood during conditions of stress (e.g. haemorrhagic shock: Ginsburg,1968). In the present experiments, the hepatic glucose release following vasopressin administration accounted for only about 70% of the reduction in hepatic glycogen concentration which occurred over this period. The balance of glucose-1-phosphate produced by glycogen breakdown may have entered the glycolytic pathway, the C_2 units thus produced being directed principally towards fatty acid synthesis or CO_2 production.

Vasopressin-induced hepatic glucose release in perfusion

was found to be diminished following adrenal ablation. This diminution of glucose release was observed at all circulating concentrations of the hormone which were sub-maximal for glucose release in the perfused livers of fed normal rats. Vasopressin-stimulated glucose release from the perfused livers of adrenalectomized rats only reached the level observed in the livers of sham-operated control rats in the presence of I $mU.ml^{-I}$ circulating vasopressin. When adrenalectomized rats were pretreated with subcutaneous cortisol ($10mg.kg^{-1}$) 4h. prior to liver perfusion, the glucose-mobilising effect of vasopressin ($400\ \mu U.ml^{-1}$) was restored towards the control value. Thus, the steroid dependence of vasopressin-induced glycogenolysis appears to resemble that of the adrenalin and glucagon stimulated processes as reported by Exton et.al., (1972).

4.2.3 The Role of the Adrenal Gland in the Regulation of Hepatic Gluconeogenesis.

Having established a role for adrenal corticoids in the regulation of hepatic glycogen metabolism, it was of interest to study the influence of the adrenal cortex upon hepatic gluconeogenesis.

Reports in the literature are divided as to the importance of adrenal steroids in the control of gluconeogenesis in livers perfused without added hormones. This inconsistency may be partially explained by the observation of Haft et. al. (1972), that a reduced hepatic capacity for gluconeogenesis in the adrenalectomised rat can only be demonstrated in the presence of large, non-physiological concentrations of substrates. In the present study, the influence of adrenalectomy upon basal and vasopressin-stimulated gluconeogenesis has been studied in the perfused livers of fasted rats. Perfusions were performed in the presence of 8 mM lactate (a substrate concentration which may occur in the hepatic portal vein following a glucose feed) and 2 mM pyruvate.

The present results tend to confirm the view of Haft et. al. (1972), in that basal gluconeogenesis was not influenced by adrenalectomy in the subsequently perfused livers of rats. Thus it seems likely that the many reported changes in the hepatic activities of gluconeogenic enzymes following adrenalectomy or steroid treatment (Weber et. al., 1964) may not necessarily influence the rate of basal hepatic gluconeogenesis in the rat.

Vasopressin enhanced the rate of glucose release from the livers of rats perfused with exogenous lactate and pyruvate (figure 18). This result confirms the findings of an earlier study by Hems and Whitton (1973). In the present experiments the rate of glucose release from the perfused liver was increased by about 50% in the presence of

vasopressin (400 $\mu U.ml^{-1}$). Measurements of hepatic glycogen concentrations revealed that the glucose release was not attributable to net hepatic glycogenolysis (results not shown).

The relationship between the hormone concentration and the rate of vasopressin-stimulated gluconeogenesis in the perfused livers of fasted rats was unaffected following adrenal ablation (figure 19). In this respect, the stimulation of hepatic gluconeogenesis by vasopressin is in contrast to that induced by glucagon or catecholamines, where a permissive role for adrenal corticosteroids has been postulated (N.Friedmann et.al.,1967, 1968; Exton et.al.,1972a). The possibility that this contrast reflects a qualitative difference in the mechanisms by which these three hormones exert their similar effects upon hepatic metabolism is discussed in a later section.

In general, the results discussed in this section suggest that the alterations in hepatic carbohydrate metabolism following adrenal ablation are unlikely to be a major factor influencing the changes which occur in hepatic lipid metabolism following adrenal ablation. Thus, while adrenalectomy caused a diminution in hormone-induced hepatic glycogen breakdown, there is no evidence that the availability of hepatic glycogen as a carbon source for fatty acid synthesis might be reduced in the conditions of liver perfusion used in the present study. As regards the synthesis of new glucose from lactate, while the glucagon or adrenalin-stimulated processes may be diminished following adrenalectomy, basal gluconeogenesis in the presence of physiological substrate concentrations is

probably unaffected by adrenal ablation. Thus it is unlikely that the availability of lactate carbon for hepatic fatty acid synthesis would be reduced following adrenalectomy in the present study. A more fundamental impairment of fatty acid synthesis and esterification, such as that which might result directly from diminished concentrations of circulating insulin, seems to provide a more plausable explanation for the effects of adrenalectomy upon these processes.

4.3 The Mechanism by which Vasopressin Influences Hepatic Metabolism.

Some of the experiments discussed in the last section serve to illustrate the basic characteristics of the action of vasopressin upon hepatic metabolism. The superficial similarity between these actions, and those of adrenalin and glucagon in the liver is striking, and the experiments discussed in this section were designed to investigate the mechanistic basis of this similarity. It is widely accepted that glucagon and adrenalin enhance hepatic glucose release in the fed state by activating glycogen phosphorylase a, and the role of vasopressin in the control of this enzyme's activity is discussed later. Other reactions where hormones may exert a regulatory effect upon hepatic glucose release remain more obscure. With a view to clarifying the mechanism by which vasopressin stimulates hepatic glucose output, the cellular concentrations of metabolic intermediates were measured in both fed and fasted states following vasopressin administration.

4.3.1 The Influence of Vasopressin upon the Intracellular Concentrations of Hepatic Metabolites.

The changes in the tissue concentrations of metabolic intermediates which occurred following vasopressin treatment were in many respects similar in the perfused livers

of fed and fasted rats. Thus, in both cases, vasopressin treatment resulted in diminished hepatic levels of pyruvate, unchanged concentrations of lactate, and elevated levels of the intermediates of the gluconeogenic pathway between phosphoenol pyruvate and glucose. It is clear that vasopressin does not increase the rate of hepatic gluconeogenesis merely by diminishing the flux through other pathways, since such a diminution would result in elevated levels of pyruvate.

The metabolite profiles shown in figures 21a and 22a both exhibit a 'crossover point' between pyruvate and phosphoenol pyruvate. A similar effect has also been reported in perfused livers treated with adrenalin, glucagon or cyclic AMP (Exton and Park, 1969). This result indicates a stimulation of the conversion of pyruvate to phosphoenol pyruvate. This could reflect an activation of pyruvate-carboxylase, PEPCK or both. In the experiments described here, an attempt has been made to calculate the intracellular concentration of oxalacetate from the malate dehydrogenase equilibrium. The results of this calculation indicate that the intracellular concentration of oxalacetate may be diminished following vasopressin treatment in the fasted state. No effect of the hormone upon oxalacetate concentrations were observed in the fed state. It seems likely that PEPCK activity was stimulated by vasopressin in the fed state, however the interpretation of the results from fasted animals is difficult since the high circulating concentrations of lactate were poorly controlled in these experiments. The observed 'crossover point' between pyruvate and phosphoenol-pyruvate might result from a reduction in

glycolytic flux secondary to an inhibition of pyruvate kinase. However, there are no reports that this enzyme may exert a regulatory role in the liver, and in the present study vasopressin failed to influence lactate concentrations in the liver.

A major effect of vasopressin upon hepatic intermediate concentrations in the fasted state was the marked diminution of hepatic glucose-6-phosphate concentrations in the presence of elevated levels of fructose -1, 6 - diphosphate and glucose. This change was not observed in fed preparations. This effect of vasopressin is suggestive of an activation of the enzyme glucose-6-phosphatase. Exton and Park (1966) have reported that glucagon enhances the conversion of glucose-6-phosphate to glucose in the perfused livers of fasted rats. Veneziale (1972) has demonstrated enhanced incorporation of glyceraldehyde and dihydroxyacetone into glucose in the livers of fasted rats perfused with glucagon and quinolate (which blocks the PEPCK reaction). Thus it seems possible that glucagon shares with vasopressin the ability to enhance hepatic glucose-6-phosphatase activity in the fasted state. If, as has been suggested by Nikkila and Ojala (1964), glycerol is a major precursor of glucose in the fasting rat, then the activation of glucose-6-phosphatase by the stress hormone vasopressin could have considerable physiological significance.

It is possible that the predicted reduction in the hepatic concentration of glucose-6-phosphate resulting from glucose-6-phosphatase activation in the perfused livers of fed rats could

have been masked by increased glucose-6-phosphate production due to vasopressin stimulated glycogenolysis. Further experiments are required in order to clarify the direct influence of vasopressin and other hormones upon glucose-6-phosphatase activity in vivo.

Struck et.al.,(1965) have suggested that the stimulation by glucagon of hepatic gluconeogenesis may be secondary to the activation of hepatic triacylglycerol lipase and the consequent increase in the hepatic concentration of free fatty acids. The failure of vasopressin to influence hepatic fatty acid synthesis in the present study argues against the activation of an hepatic lipase by this hormone. This follows because the increased intracellular concentrations of free fatty acids which would be expected to accrue following such an activation would be likely to inhibit de novo fatty acid synthesis.

In figures 21b and 22b are shown the changes that occur in the hepatic concentrations of some metabolites concerned with the entry of amino-acids, via the tricarboxylic acid cycle, into the gluconeogenic pathway. In both fed and fasted states, vasopressin treatment resulted in a reduction of the hepatic concentration of α-ketoglutarate in the presence of elevated or unchanged malate concentrations respectively. These changes are suggestive of enhanced oxidative decarboxylation of α-ketoglutarate within the tricarboxylic acid cycle. The increase in the hepatic concentration of aspartate following vasopressin treatment, together with the unchanged citrate and undiminished malate concentrations might reflect increased

aspartate production by the glutamate-oxalacetate transaminase reaction. Activation of this reaction would also maintain the supply of α-ketoglutarate for oxidative decarboxylation. Thus it is possible that the vasopressin-induced changes in the intracellular concentrations of these metabolites reflect enhanced transamination of glutamate from endogenous protein. This enhanced transamination could be secondary to the increased oxidative decarboxylation of α-ketoglutarate discussed above. The increased intracellular levels of aspartate could become available to the gluconeogenic pathway by transamination to oxalacetate in the cytosol, (thus contributing to the diminution of intracellular α-ketoglutarate concentrations). Alternatively, the aspartate could enter the urea cycle, and the fumarate thus produced be converted to oxalacetate by the action of fumarase and malate dehydrogenase. The overall effect of these changes would be to enhance the availability of oxalacetate for conversion to glucose.

The influence of vasopressin upon the hepatic concentrations of these metabolites is similar to that observed by Ui et. al. (1973a) in the glucagon-treated perfused liver. These workers measured the incorporation of ^{14}C from (^{14}C)-glutamate into various intermediates, and also changes in their intracellular concentrations, in response to glucagon. They concluded that glucagon stimulated the entry of glutamate into the gluconeogenic pathway by means of the reactions discussed above. The observation that vasopressin and glucagon exert similar effects on the hepatic metabolism of these intermediates suggests that these two hormones could influence hepatic gluconeogenesis via a common mechanism.

The calculated ratios of oxidised to reduced pyridine nucleotides are shown in table 13, and it is clear that these ratios are diminished following vasopressin treatment in both the fed and fasted states. Williamson (1966) has suggested that in fasted rats, the intracellular concentration of acetyl CoA may be sufficiently high to ensure that the pyruvate carboxylase reaction is not rate controlling. If this is so, then elevated levels of cytoplasmic NADH could enhance the activity of the triose-phosphate dehydrogenase reaction, thus causing a rise in the overall rate of gluconeogenesis.

Veech et.al.,(1969) have suggested that the cytoplasmic NADP couple is more oxidised in states where there is rapid synthesis of fatty acids. In the case of glucagon, (which has been reported to inhibit hepatic fatty acid synthesis in several species: Goodridge, 1973; Meikle et.al., 1973), the cytoplasmic $NADP^+$ / NADPH ratio calculated from the data of Ui et.al.,(1973b)falls from 0.015 to 0.006 following hormone treatment in the fed state. In the present study, this couple was markedly reduced following vasopressin treatment in both fed and fasted states. It might be suggested that accumulation of cytoplasmic NADPH could reflect diminished rates of hepatic fatty acid synthesis in response to vasopressin. However, the current experiments failed to reveal any effect of vasopressin upon fatty acid synthesis from ^3H_2O or (^{14}C)-lactate in conditions which are

optimal for fatty acid synthesis in the perfused liver (Salmon and Hems, 1974). The failure of vasopressin to influence lipogenesis in the rat is in contrast to it's action in the mouse, where vasopressin induced glycogen breakdown is paralleled by an inhibition of fatty acid synthesis (Ma and Hems, 1975). It is therefore clear that there is a species difference with respect to the action of vasopressin upon hepatic lipogenesis.

The oxidation of exogenous fatty acid, as measured by the release of ketone bodies into the perfusate was also uninfluenced by the presence of vasopressin ($1mU.ml^{-1}$) in the perfusion medium. The failure of vasopressin to influence ketogenesis in the liver represents another contrast in the hepatic actions of adrenalin and glucagon, since relatively large doses of the latter hormone have been reported to stimulate ketogenesis in the perfused liver (Heinberg et.al., 1969).

4.3.2 The Influence of Insulin upon Vasopressin-induced Glucose Release from the Perfused Liver.

The glucose-mobilising effect of a saturating concentration of circulating vasopressin ($400\ \mu U.ml^{-1}$) was reduced by about 65% when insulin ($5mU.ml^{-1}$) was present in the perfusion medium (figure 20). In this respect, the glycogenolytic effect of vasopressin is similar to that of glucagon, since the effect of low concentrations of this

latter hormone has been reported to be greatly diminished in the presence of insulin (Glinsmann and Mortimore, 1968). Insulin has also been reported to diminish basal glucose release in the perfused liver (Mortimore et.al., 1967), and to antagonise the stimulation of hepatic gluconeogenesis by glucagon, adrenalin or cyclic AMP (Claus and Pilkis, 1975). Mackrell and Sokal (1969) have reported that insulin concentrations as low as 140 $\mu U.ml^{-1}$ can inhibit the action of 10^{-10}M glucagon on hepatic glucose output and produce a decrease in phosphorylase activity. Furthermore, these workers demonstrated that insulin concentrations between 140-3000 $\mu U.ml^{-1}$ caused a reduction in basal phosphorylase activity in the liver.

The glucose mobilising effect of vasopressin has been associated with an increase in the activity of hepatic glycogen phosphorylase a (Hems et.al., 1975). It is therefore possible that insulin might exert it's inhibitory effect upon vasopressin-induced glycogenolysis by directly influencing the activation of glycogen phosphorylase a by vasopressin.

4.3.3 The Lack of a Role for Cyclic Nucleotides in the Hepatic Actions of Vasopressin.

It has been suggested that insulin may diminish the activity of glycogen phosphorylase, and antagonise the effect of glucagon upon hepatic glucose release by reducing the intracellular concentrations of cyclic AMP (Illiano and Cuatrecasas, 1972; Pilkis et.al., 1975). Indeed, until recently,

there has been widespread acceptance of the theory that all short-term hormonal effects upon the liver are mediated by this nucleotide. Thus, it has been suggested that the elevation by adrenalin and glucagon of hepatic rates of glycogenolysis and gluconeogenesis, and the stimulation by the latter hormone of hepatic ureogenesis and ketogenesis all involve the action of cyclic AMP as a 'second messenger' (Exton et.al., 1971b; Robison et.al., 1971). De Lorenzo et.al., (1973) have reported that the vasopressin-induced elevation of Na^+ permeability in the toad bladder is associated with an increase in the intracellular levels of cyclic AMP. A similar effect upon cyclic AMP concentrations has been reported to accompany the antidiuretic actions of vasopressin and oxytocin (Chase & Aurbach, 1968 ; Schultz et.al. 1972).

In the present study, vasopressin failed to elevate the hepatic concentration of cyclic AMP when injected into the hepatic portal vein in vivo. Both glucagon and adrenalin caused an elevation of hepatic cyclic AMP concentrations when administered in this fashion. All three hormones increased the activity of hepatic glycogen phosphorylase in liver samples taken from these rats (Hems et.al., 1975 and figure 27). Thus it is clear that vasopressin can increase the activity of hepatic glycogen phosphorylase without causing a measurable rise in the intracellular concentration of cyclic AMP.

It has been suggested that hormonal stimulation of adenylate cyclase may result in elevation of only a small 'active pool' of cyclic AMP without affecting the much larger inactive or bound pool (Exton and Park, 1972). It is

thought that increases in the size of the active pool are reflected by enhanced cyclic AMP release into the circulation (Okajima and Ui, 1976). In the present study, vasopressin failed to influence cyclic AMP release into the effluent perfusate although the hormone induced hepatic glucose release which was of the same order as that caused by adrenalin. In contrast, glucagon and adrenalin produced the expected increments in hepatic cyclic AMP release. Vasopressin also failed to elevate hepatic cyclic AMP release from the liver of a fasted adrenalectomized rat perfused in the presence of theophylline, conditions in which the hormone sensitivity of the adrenalin sensitive adenylate kinase is reported to be enhanced (Bitensky et.al.,1970; Exton et.al., 1971b) These results suggest that the hepatic actions of vasopressin are not mediated by cyclic AMP. This conclusion is in keeping with the observation that vasopressin exerts no effect upon the activity of hepatic adenylate cyclase, (personal communication from M.Rodbell), or protein kinase (Keppens and De Wulf, 1975).

Sherline et.al. (1972) first demonstrated that adrenalin-induced glycogenolysis in the perfused liver was not influenced by β-adrenergic inhibitors capable of preventing any elevation of hepatic cyclic AMP concentration. Tolbert et. al. (1973) have demonstrated that while propranolol blocked adrenalin stimulated cyclic AMP production in hepatocytes, the drug had no effect upon the hormonal stimulation of gluconeogenesis. These workers also showed that low concentrations of dihydroergotamine decreased adrenalin-stimulated

gluconeogenesis but exerted no effect upon the cyclic AMP-or glucagon-stimulated processes. Similarly, isoproterenol elevated the concentration of cyclic AMP in hepatocytes, but did not influence basal or adrenalin-induced gluconeogenesis. This work demonstrates that the stimulation of hepatic gluconeogenesis by adrenalin is an α-adrenergic effect, which can occur independently of any rise in the intracellular concentration of cyclic AMP. Okajima and Ui (1976) have demonstrated that low doses of glucagon or adrenalin can activate hepatic glycogen phosphorylase and induce glycogenolysis in vivo, without increasing cyclic AMP synthesis from (^{3}H)-adenine, or the release of this nucleotide from the liver. Recently, Van de Werve et. al. (1977) have reported that phenylephrine, isoproterenol and vasopressin can all enhance the activity of glycogen phosphorylase in hepatocytes without influencing the activity of the cyclic AMP-sensitive phosphorylase kinase. Thus vasopressin, both α- and β-adrenergic agonists and possibly even glucagon may influence hepatic metabolism via cyclic AMP-independent mechanisms.

Several workers have also reported that adrenalin-induced lipolysis in adipocytes, or lipid micelles, can occur in the absence of elevated cyclic AMP levels and without increased protein kinase activity (Fain, 1973; Okuda et.al., 1974; Saito et.al., 1974a,b).

It has been suggested that guanosine-3',5'-cyclic monophosphate (cyclic GMP) may mediate the metabolic actions of some hormones in a similar fashion to cyclic AMP (Goldberg et. al., 1969; Thompson et. al., 1973). Exton et. al. (1971b) have

shown that exogenous cyclic GMP can exert similar effects to those of cyclic AMP in the perfused liver if it is present relatively high concentrations. Of the four hormones tested in the present study (glucagon, adrenalin, vasopressin and insulin), all failed to influence significantly the hepatic concentration or release of cyclic GMP (assays performed by Dr K.Siddle, data presented with permission in table 16) The concentrations of cyclic GMP which were detected in the perfused liver were much smaller than either basal or hormone stimulated cyclic AMP concentrations. It seems unlikely that these very low concentrations of cyclic GMP could have any role in the regulation of hepatic glycogenolysis unless the nucleotide exists in localised pools of much higher concentration. This follows from the work of Exton et.al., (1971c) who demonstrated that exogenous cyclic GMP is only about $\frac{1}{2}$ - $\frac{1}{3}$ as potent as cyclic AMP as an inducer of glycogenolysis in the perfused liver.

4.3.4 The Role of Extracellular Ca^{++} Ions in Vasopressin Action upon the Liver

There have been many reports concerning the Ca^{++} dependence of both basal and hormone stimulated gluconeogenesis in isolated hepatocytes (Zahlten et.al.,1974; Tolbert and Fain, 1973; Pilkis et.al.,1975). The general conclusion from these studies is that rates of basal gluconeogenesis and the glucagon-,adrenalin-,or cyclic AMP-

stimulated processes are considerably reduced in the absence of Ca^{++} ions. The response of the hepatocytes could be restored to normal by the addition of Ca^{++} to the incubation medium. The precise role of Ca^{++} in the activation of hepatic gluconeogenesis remains obscure, but Pilkis et.al.,(1975) have demonstrated that glucagon exerts a rapid stimulatory effect upon the flux of Ca^{++} between hepatocytes and their incubation medium.

In the course of experiments designed to elucidate the role of extracellular Ca^{++} ions in vasopressin-stimulated glucose release from the perfused liver, it became clear that this process was exquisitely temperature sensitive. Hence basal and vasopressin stimulated glucose release from the perfused liver were studied in the presence and absence of added Ca^{++} over a range of temperatures. The temperature sensitivity of glucose release from the perfused liver was not influenced by the addition of vasopressin to the perfusate, but it was increased in the absence of added Ca^{++}. The incremental glucose release produced by vasopressin was similarly not influenced by temperature, but it was reduced by about 40% in the absence of added Ca^{++}. This observation was confirmed in isolated liver cells by Stubbs et.al.,(1976) who showed that in the absence of Ca^{++}, vasopressin exerted no stimulatory effect upon glucose release from hepatocytes prepared in Ca^{++}-free medium. In the same study, glucagon- and adrenalin-stimulated glycogenolysis in isolated hepatocytes was not influenced by Ca^{++} ion depletion. This result was confirmed by

Pointer et.al.(1976).However,changes in intracellular Ca^{++} levels have been reported to influence glucagon stimulated hepatic glycogenolysis by Clarke et.al.,(1974).These workers showed that the effect of glucagon on this process was diminished in the presence of a Ca^{++} ionophore. Clarke et. al. (1974) suggested that the antagonism by insulin of glucagon-stimulated hepatic glucose release might be mediated by an increase in intracellular Ca^{++}.

It is clear that the Ca^{++}-sensitivity of vasopressin-induced glycogenolysis represents another major difference between the mode of action of this hormone and those of adrenalin and glucagon. It seems likely that Ca^{++} is implicated in the activation by vasopressin of hepatic glycogen phosphorylase. The involvment of Ca^{++} in this process may be related to the potent activation of hepatic glycogen phosphorylase kinase by Ca^{++} (Khoo and Steinberg (1975).

4.3.5 A Summary of the Information Currently Available Concerning the Mechanism by which Vasopressin Influences Hepatic Metabolism.

The actions of vasopressin upon hepatic carbohydrate metabolism do not conform to the cyclic AMP-mediated pattern of events which has been accepted as the basis for the hepatic actions of glucagon and β-adrenergic agonists. With regard to gluconeogenesis, vasopressin treatment (like glucagon treatment) leads to enhanced conversion of pyruvate to

phosphoenol pyruvate and oxidative decarboxylation of ∝-ketoglutorate. Vasopressin-stimulated gluconeogenesis is not steroid dependent and may involve a stimulation of hepatic glucose-6-phosphatase activity. The glucose-mobilising effect of vasopressin in the fed state has been shown to be associated with an increase in glycogen phosphorylase a activity. This activation of glycogen phosphorylase is not mediated by cyclic AMP. However the vasopressin-induced release of hepatic glucose is Ca^{++} dependent, and diminished following adrenalectomy. Further experiments are required to elucidate the precise mechanism(s) by which vasopressin (and the various adrenergic agonists) influence hepatic metabolism. A possible role for ion transport across membranes merits particular attention.

5 References

Adam,H.(1963) in: Methods of Enzymic Analysis,p.573 (Ed. Bergmeyer H.V.), Academic Press, (New York).

Addison,T.(1855) On the Constitution and Local Effects of Disease of the Suprarenal Capsules. S.Highley,(London).

Baillie, L.A.(1960) Int.J.Appl.Radiat.Isotop. 8,1.

Barnes,R.H., Miller,E.S and Burr,C.O. (1941) J.Biol.Chem. 140, 233.

Baron,D.N. and Roberts,P.(1963) J.Physiol.(London) 165,219.

Basso, L.V. and Havel,R.J.(1970) J.Clin.Invest. 49,537.

Beatty,C.H.(1945) Am.J.Physiol. 143, 579.

Bendall,J.R. and Lehmann,H,(1942) Biochem.J. 36, XIV.

Bergen,S.S., Sullivan,R., Hilton,J.G., Willis,S.W. and van Hallie,T.B.(1960) Am.J.Physiol. 199, 136.

Berliner,D.C., Leong,G.F., Cazes,D.M. and Berliner,M.L.(1962) Am.J.Physiol. 202,420.

Bernhard,K. and Bullett,F. (1943) Helv.Chim.Acta. 26, 1185.

Bickerstaffe,R.and Annison,E.F.(1970) Biochem.J. 118, 433.

Bierry,H. and Malloizel,L. (1908) C.R.Soc.Biol.(Paris) 65, 232

Bitensky,M.W.,Russell,V. and Blanco,M (1970) Endocrinology 86, 154.

Bizzi,A., Facconi,M.T. and Medea,A.(1972) Pharmacology 7, 216.

Bluell,M.V.,Anderson,I.A. and Strauss,M.B. (1936) Am.J. Physiol. 116, 274.

Bondy,P.K.(1949) Endocrinology 45, 605.

Borchardt,L. (1908) Dtsch.Med.Wschr. 34, 946.

Brady,R.O., Lukens,F.D.W. and Gurin,S.(1951) J.Biol.Chem.193, 459.

Braun,T. and Hechter,O. (1970) Proc.Natl.Acad.Sci.(USA) 66, 995.

Britton,S.W. and Silvette,H.(1932) Am.J.Physiol. 100, 693.

Britton,S.W. and Silvette,H. (1934) Am.J.Physiol. 107, 190.

Brown,B.L., Albano,J.D.M., Ekins,R.P., Sgherzi,A.M. and Tampion,W. (1971) Biochem.J. 121,561.

Browne,J.S.L., Schenker,V. and Stevenson,J.A.E.(1944) J.Clin. Invest. 23, 932.

Brunengraber,H., Sabine,J.R., Boutry,M. and Lowenstein,J.M. (1972) Arch.Biochem.Biophys. 150, 392.

Brunengraber,H., Boutry,M. and Lowenstein,J.M. (1973) J.Biol. Chem. 249, 2028.

Bucher,T. and Hohorst,H.J. (1963) in: Methods of Enzymatic Analysis, p.246.(Ed.Bergmeyer H.V.), Academic Press,(New York).

Butcher,R.W. and Baird,C.E. (1968) J.Biol.Chem.243, 1713.

Carey,L.C., Sapira,J.D. and Curtin,R.A.(1972) Bull.Soc.Int. Chir. 5, 393.

Chase,L.R. and Aurbach,G.D. (1968) Science 159, 545.

Celander,O. (1954) Acta.Physiol. Scandinav. 32 Suppl, 116.

Chen,R.F. (1967) J.Biol.Chem 242, 173.

Chiu,C.Y. and Needham,D.M. (1950) Biochem.J. 46, 114.

Clarke,P., Kissebah,A., Vydelingum,N., Hope-Gill,H., Tulloch,B. and Fraser,R. (1974) Horm.Metab. Res. 6, 525.

Claus,T.H. and Pilkis,S.J. (1976) Biochim. Biophys.Acta. 421,246.

Cline,T.N., Cole,J.W. and Holden,W.D. (1953) Surg.Gynecol. Obstet 96, 674.

Cope,C.L. and Pearson,J.(1965) J.Clin.Path. 18,82.

Cooper,C.E. and Nelson,D.H. (1962) J.Clin.Invest. 41,1599.

Corbin,J.D. and Park,C.R. (1969) Fed.Proc. 28, 702.

Corey,E.L. and Britton,S.W. (1941) Am.J. Physiol 131, 783.

Craig, J.W., Rall,T.W.and Larner,J. (1969) Biochim.Biophys.Acta. 177, 213.

Cuthbertson,D.P. (1930) Biochem.J. 24, 1244.

Czok,R. and Eckert,L. (1963) in: 'Methods of Enzymatic Analysis', p.224, (Ed.Bergmeyer H.V.) Academic Press, (New York).

De Gasquet,P., Pequignot-Planche,E., Tonnu,N.T. and Diaby,F.A. (1975) Horn.Metab.Res. 7, 152.

De.Lorenzo, R.J., Walton,K.G., Curran,P.F. and Greengard,P.(1973) Proc.Natl.Acad.Sci.(USA) 70, 880.

Diamant,Y.Z., Neuman,A. and Shafrir,E. (1975) Biochim Biophys. Acta. 385, 257.

Diamant,S. and Shafrir,E. (1975) Eu.J.Biochem. 53, 541.

Di Luzio,N.R., Shore,M.L. and Zilversmit,D.B. (1954) Metabolism Clin. and Exptl. 3, 424.

Edwards,P.A. (1973) J.Biol.Chem. 248, 2912.

Eidinoff,M.L., Perri,G.C., Knoll,J.E., Marano,B.J. and Arnheim,J. (1953) J. Amer.Chem.Soc. 75, 248.

Eisenstein,A.B., Berg,E. Goldenberg,D. and Jensen,B.(1964) Endocrinology 74, 123.

Eisenstein,A.B., Spencer,S., Flatness,S. and Brodsky,A.(1966) Endocrinology 79, 182.

Evans,G.T. (1936) Am.J.Physiol. 114, 297.

Evans,G. (1941) Endocrinology 29, 731.

Exton,J.H. and Park,C.R. (1965) J.Biol.Chem. 240, PC 955.

Exton,J.H. and Park,C.R. (1966) Pharmacol.Rev. 18, 181.

Exton,J.H. and Park,C.R. (1969) J.Biol. Chem. 244, 1424.

Exton,J.H. and Park,C.R. (1972) in: 'Handbook of Physiology', Section 7, Volume 1,p437,American Physiological Society.

Exton,J.H., Mallette,L.E., Jefferson,L.S., Wong,E.H.A., Friedmann,N., Miller,T.B. and Park,C.R. (1970) Rec.Prog. Horm.Res. 26, 411.

Exton,J.H., Lewis,S.B., Ho,R.J., Robinson,G.A. and Park,C.R. (1971a) Ann.N.Y. Acad.Sci. 185, 85.

Exton,J.H., Robinson,G.A., Sutherland,E.W. and Park,C.R. (1971b) J.Biol.Chem. 246, 6166.

Exton,J.H., Hardman,J.G., Williams,T.F., Sutherland,E.W. and Park,C.R. (1971c) J.Biol.Chem. 246, 2658.

Exton,J.H., Friedmann,N., Wong,E.H., Brineaux,J.P.,Corbin,J.P. and Park,C.R. (1972a) J.Biol.Chem. 247, 3579.

Exton,J.H., Robison,G.A. and Sutherland,E.W. (1972b) in: Handbook of Physiology,Section 7 (Endocrinology, Greep R.O. and Astwood E.B., eds), Vol.1, p.437, American Physiological Society.

Exton,J.H., Harper,S.C., Tucker,A.L., Flagg,J.L. and Park,C.R. (1973) Biochim. Biophys.Acta. 329, 41.

Fain,J.N. (1973) Mol.Pharmacol. 9, 595.

Fain,J.N. and Wilhelmi,A.E. (1962) Endocrinology 71, 541.

Fain, J.N., Scow,A.O. and Chernick,S.S. (1963) J.Biol.Chem. 238, 54.

Folch,J., Ascoli,I., Lees,M., Meath,J.A. and Le Baron,F.N.(1951) J.Biol.Chem. 191, 833.

Folch,J., Lees,M. and Sloane-Stanley,G.N.(1957) J.Biol.Chem. 226, 497.

Forsling,M.(1975) J.Physiol (London) 241, 35P.

Freeman,C.P. and West,D. (1966) J.Lipid Res. 7, 324.

Friedmann,B., Goodman,E.H. and Weinhouse,S. (1965) J.Biol. Chem. 240, 3729.

Friedmann,B., Goodman,E.H. and Weinhouse,S.(1967) Endocrinology 81, 486.

Friedmann,N., Exton,J.H. and Park,C.R. (1967) Biochem.Biophys. Res.Comm. 29, 113.

Friedmann,N., Exton,J.H. and Park,C.R. (1968) Fed.Proc. 27, 625.

Fry, E.G.(1937) Endocrinology 21, 283.

Garrison,J.C. and Haynes,R.C.Jr. (1973) J.Biol.Chem. 248, 5333.

Gellhorn,A. and Benjamin,W. (1966) Ad.Enz.Regul. 4, 19.

Ginsburg,M. (1968) in: Handbook of Experimental Pharmacology, Vol 23, (Ed.Berde,B.), p.286, Springer Verlag.

Ginsburg,M. and Heller,M. (1953) J.Endocrinol. 9, 274.

Glinsmann,W.H.and Mortimore,G.E. (1968) Am.J.Physiol. 215, 553.

Glock,G.E and McLean,P. (1955) Biochem.J. 61, 390.

Goldberg,N.D., Deitz,S.B. and O'Toole,A.G. (1969) J.Biol.Chem. 244, 4488.

Goldberg,N., Haddox,M.K., Hartle,D.K. and Hadden,J.W. in: Proceedings of the Fifth International Congress of Pharmacology, San Francisco, 1972,(1973) Vol 5, p.146, Karger (Basel).

Goodridge,A.G. (1973) J.Biol.Chem. 248, 1924.

Haft,D.E. (1967) Am.J.Physiol. 213, 219.

Haft,D.E. and Miller L L. (1956) Biochim.Biophys.Acta. 19, 386.

Haft,D.E. and Miller,L.L. (1958) Am.J. Physiol. 192, 33.

Haft,D.E., Tennen,E. and Mehtalia,J.(1972) Am.J.Physiol. 222, 365.

Hays,A.P. and Hill, R.B.(1965) Biochim.Biophys.Acta. 98, 646.

Heidenreich,O. and Reus,E.(1962) Naunyn-Schmiedebergs Arch.exp. Path.Pharmak. 243, 136.

Heidenreich,O., Kook,Y., Baumeister,L. and Keller,P.(1963) Naunyn-Schmiedebergs.Arch.exp.Path.Pharmak. 245, 321.

Heimberg,M.(1966) Biochim.Biophys.Acta.125,252.

Heimberg,M., Weinstein,I. and Kohout,M.(1969) J.Biol.Chem. 244, 5131.

Hems,D.A. and Whitton,P.D.(1973) Biochem J. 136, 705.

Hems,D.A., Whitton,P.D. and Taylor,E.A.(1972) Biochem.J. 129, 529.

Hems,D.A., Rath,E.A. and Verrinder,T.R.(1975a) Biochem.J. 150, 167.

Hems,D.A., Whitton,P.D. and Ma,G.Y. (1975b) Biochim.Biophys. Acta. 411, 155.

Hems,R., Ross,B.D., Berry,M.N. and Krebs,H.A.(1966) Biochem.J. 101, 284.

Herberg,L. and Kley,H.K.(1975) Horm.Metab.Res. 7, 410.

Hendler,R.W.(1964) Anal.Biochem. 7, 110.

Henly,K.S.,Kawata,H. and Pino,M.E. Endocrinology. 73, 366.

Henning,H.V., Seiffert,I. and Seubert,W.(1963) Biochim.Biophys. Acta. 77, 345.

Hickman,P.E., Horton,B.J. and Sabine,J.R.(1972) J.Lipid Res. 13, 17.

Hilz,H., Farnowski,W. and Arend,P.(1963) Biochem.Biophys.Res. Commun. 10, 492.

Hohorst,H.J.(1963a) in 'Methods of Enzymic Analysis',p215, (Ed.Bergmeyer H.F.), Academic Press,(New York).

Hohorst,H.J.(1963b) in: 'Methods of Enzymatic Analysis',p134, (Ed.Bergmeyer H.V), Academic Press,(New York).

Holmes,E. and Lehmann,H.(1940) Brit.J.Exp.Pathol.21, 196.

Hornbrook,K.R., Burch,H.B. and Lowry,O.H.(1966) Mol.Pharmacol. 2, 106.

Huber,J., Hamprecht,B., Muller,O. and Guder,W.(1972) Hoppe-Seyler's Z. Physiol.Chem. 353, 313.

Ingle,D.J.(1951) Endocrinology 29, 649.

Ingle,D.J., Prestrud,M.C. and Nezamis,J.E.(1948) Proc.Soc. Exptl.Biol.(NY) 67, 321.

Ingle,D.J.(1952) Endocrinology 8, XXIII

Illiano,G. and Cuatrecosas,P.(1972) Science 72, 906.

Imrie,C.G.(1929) J.Physiol.(London) 67, 264.

Issekutz,B.Jr. and Borkow,I. (1973) Metabolism 22, 39.

Itoh,S.M., Tsukada,M., Okuno,A. and Yoshinari,T.(1966) Jap.J. Physiol.16, 23.

Jeanrenaud,B. and Renold,A.E.(1960) J.Biol.Chem. 235, 2217.

Johnson,M.E.M., Das,N.M., Butcher,F.R. and Fain,J.N.(1972) J.Biol.Chem. 247, 3229.

Jungas,R.L.(1968) Biochemistry 7, 3708.

Katzin,B. and Long,C.N.H.(1938) Am.J.Physiol. 123, 113.

Kappers, S. and De Wulf, H.(1975) FEBS. Letters 51, 29.

Khoo,J.C. and Steinberg,D.(1975) FEBS. Letters 57, 68.

Klausner,H. and Heimberg,M.(1967) Am.J.Physiol. 212, 1236.

Kobernick,S.D. and More,R.H.(1950) Proc.Soc.Exptl.Biol.Med. 74, 602.

Koeff,G.F.,Horn,H.W.,Gemmill,C.L. and Thorn,G.W.(1941) Am.J. Physiol. 135, 175.

Krebs,H.A. and Henseleit,K.(1932) Hoppe-Seyler's Z.Physiol.Chem. 210, 33.

Krebs,H.A.,Dierks,C. and Gascoyne,T.(1964) Biochem.J. 93, 112.

Krebs,H.A.,Wallace,P.E.,Hems,R. and Freedland,R.A(1969) Biochem.J. 112, 595.

Kuo,J.F. and Greengard,P.(1969) Proc.Natl.Acad.Sci. 64, 1349.

Kyner,J.L., Levy,R.I., Soeldner,J.S.(1972) Metabolism 21, 329.

Lamberts,S.W.J., Timmerjnans,H.A.T., Kramer-Blankestijn,M. and Birkenhager,J.C.(1975) Metabolism 24, 681.

Lardy,H.A.,Foster,D.O., Young,J.W.,Shrago,E. and Ray,P.D.(1965) J. Cell.Comp.Physiol. 66, Suppl 1, 39.

Lauwerys,R.R.(1969) Anal.Bioch. 32, 331.

Leblond,C.P., Nguyen-Van Thoai and Segal,G.(1939) Compt.Rand. Soc.Biol. 130, 1557.

Leboeuf,B., Renold,A.E. and Cahill,G.F.Jr.(1962) J.Biol.Chem. 237, 988.

Levy,A.C. and Ramey,E.R.(1959) Endocrinology 64, 586.

Levey-Simpson,S(1932) Quart.J.Med. 1, 99.

Lewis,R.A., Kuhlman,D., Delbue,C. Koepf,G.F. and Thorn,G.W. (1940) Endocrinology 27, 971.

Linder,C. and Migliorini,R.H.(1974) Diabetes 23, 143.

Lipkin,D., Cook,W.H. and Markham,R.(1959) J.Amer.Chem.Soc. 81, 6198.

Lipsett,M.N. and Moore,F.J.(1951) J.Biol.Chem. 192, 743.

Livingston,J.N. and Lockwood,D.H.(1975) J.Biol.Chem. 250, 8353.

Long,C.N.H., Katzin,B. and Fry,E.G.(1940) Endocrinology 26,309.

Long,C.N.H., Fry,E.G. and Bonnycastle,M.(1960) Acta Endocrinol. Suppl 51, 35, 819.

Loten,E.G. and Sneyd,J.G.T.(1970) Biochem.J. 120, 187.

Lowenstein,J.M. and Kornacker,M.S.(1964) Biochim.Biophys.Acta. 84, 490.

Ma,G.Y. and Hems,D.A.(1975) Biochem.J. 152, 389.

MacKay,E.M. and Carne,H.O.(1938) Proc.Soc.Exptl.Biol.Med. 38, 131.

Mackrell,D.J. and Sokal,J.E.(1969) Diabetes 18, 724.

Mayes,P.A. and Topping,D.L.(1974) Biochem.J. 140, 111.

Meikle,A.W., Klain,G.J. and Hannon,J.P.(1973) Proc.Soc.Exptl. Biol.Med. 143, 379.

Mercuri,D., Peluffo,R.O. and De Tomas,M.E.(1974) Biochim. Biophys.Acta. 369, 264.

Mersmann,H.J. and Segal,H.L.(1969) J.Biol.Chem. 244, 1701.

Metcalf,L.D. and Schmitz,A.A.(1961) Anal.Chem. 33, 363.

Migeon,C.J.,Green,O.C. and Eckert,J.P.(1963) Metabolism 12, 718.

Miller,L.L.(1961) Rec.Prog.Horm.Res. 17, 539.

Miller,L.L., Bly,C.G., Watson,M.L. and Bale,W.F.(1951) J.Exp. Med. 94, 431.

Miller,T.B.Jr. Hazen,R. and Larner,J.(1973) Biochem.Biophys.Res. Comm. 53, 466.

Mirsky,I.A.(1962) Proc.Soc.Expt. Biol.(N.Y.) 110, 42.

Mirsky,I.A.(1968) Handb.Exp.Pharmacol. 23, 613.

Moellering,H. and Gruber,W.(1966) Anal.Biochem. 17, 369.

Moran,W.H., Miltenberger,F.W., Shuayb,W.A. and Zimmermann,B. (1964) Surgery 56, 99.

Mortimore,G.E., King,E.Jr.,Mondan,C.E. and Glinsmann,W.H.(1967) Am.J.Physiol. 212, 179.

Mukherjee,S.P. and Mukherjee,C.(1973) Am.J.Physiol. 224, 898.

Munck,A.(1962a) Endocrinology 68, 178.

Munck,A.(1962b) Biochim.Biophys.Acta. 48, 618.

Munck,A. and Koritz,S.B.(1960) in "First International Congress of Endocrinology", (Ed.Fuchs F.) p.821, Periodica (Copenhagen).

Muto,Y. and Gibson,D.M.(1970) Biochem.Biophys.Res.Comm. 38, 9.

Nervi,F.O. and Dietschy,J.M.(1974) Biochim.Biophys.Acta. 369, 351.

Nichol,W.K. and Goldberg,N.D.(1972) Biochim.Biophys.Acta. 279, 245.

Nikkila,E.W. and Ojala,K.(1964) Life Sci. 3, 243.

Novello,F., Gumoa,K.A. and McLean,P.(1969) Biochem.J. 111, 713

Okajima,F. and Ui,M.(1976) Arch.Biochem.Biophys. 175, 549.

Okuda,H., Saito,Y., Matsuoka,N. and Fujii,S.(1974) J.Biochem. (Tokyo) 75, 131.

Park,C.R., Lewis,S.B. and Exton,J.H.(1972) Diabetes 21, 439.

Perry,W.F. and Bowen,H.F.(1955) Am.J. Physiol. 180, 21.

Perry,W.F. and Bowen,H.F.(1956) Am.J. Physiol. 184, 59.

Pfleiderer,G.(1963) in: Methods of Enzymatic Analysis p.381, (Ed.Bergmeyer H.V.) Academic Press,(New York).

Pilkis,S.J., Claus,T.H., Johnson,R.A. and Park,C.R.(1975) J.Biol.Chem. 250, 6328.

Pointer,R.H., Butcher,F.R. and Fain,J.N.(1976) J. Biol.Chem. 251, 2987.

Rall,T.W., Wosilait,W.D. and Sutherland,E.W.(1956) J.Biol. Chem. 218, 483.

Reaven,E.P., Kolterman,O.G. and Reaven,G.M.(1974) J.Lipid Res. 15,74.

Reinecke,R.M.(1943) Am.J.Physiol. 140, 276.

Reshef,L. and Shapiro,B.(1960) Metabolism 9, 551.

Reynolds,R.D., Potter,Van R. and Pitot,H.C.(1971) J.Nutr. 101, 797.

Rittenberg,D. and Schoenheimer,R.(1937) J.Biol.Chem. 121,235.

Rhoades,R.A., Gassenheimer,L.N. and Blackburn,W.R.(1974) Proc. Soc.Exptl.Biol. Med. 145, 987.

Robinson,D.S. and Wing,D.R.(1970) Horm.Metab.Res. 2, Suppl 2, 41.

Robison,G.A., Butcher,R.W. and Sutherland,E.W.(1971) in Cyclic AMP, Academic Press (New York).

Saito,Y., Okuda,H. and Fujii,S. (1974a) J.Biochem.(Tokyo) 75,1327.

Saito,Y , Matsuoka,N., Okuda,H. and Fujii,S.(1974b) J.Biochem. (Tokyo) 76, 1061.

Salmon,D.M.W. and Hems,D.A.(1973) Biochem.J. 136, 551.

Salmon,D.M.W. and Hems,D.A.(1975) Biochem.Soc.Trans. 3, 510.

Salmon,D.M.W., Bowen,N.L. and Hems,D.A.(1974) Biochem.J. 142, 611.

Sayers,G., Sayers,M.A., Liang,T.Y. and Long,C.N.H.(1945) Endocrinology 37, 96.

Schaeffer,L.D., Chenoweth,M. and Dunn,A. (1969) Biochim. Biophys.Acta. 192, 292.

Scheving,L.E. and Pauly,J.E.(1966) Am.J.Physiol. 210, 1112.

Schiffer,F. and Wertheimer,E.(1947) J. Clin.Endocrinol. 5, 147.

Schoenheimer,R. and Rittenberg,D. (1935) J.Biol.Chem.111, 163.

Schoenheimer,R. and Rittenberg,D. (1937) J.Biol.Chem. 114, 381.

Schotz,M.C., Masson,G.M.C. and Page,I.H.(1959) Proc.Soc. Exptl. Biol.Med. 101, 159.

Schultz,G., Jakobs,K.H., Bohme,E. and Schultz,K.(1972) Eu.J. Biochem. 24, 520.

Seckel,H.P.G. (1940) Endocrinology 26, 97.

Selye,H. and Dosne,C. (1940) Am.J.Physiol. 128, 729.

Shafrir,E. and Kerpel,S. (1964) Arch.Biochem.Biophys. 105, 237.

Shafrir,E., Sussman,K.E. and Steinberg,D. (1960) J.Lipid Res. 1, 459.

Shaw,J.E. and Ramwell,P.W.(1968) J.Biol.Chem.243, 1498.

Shaw,J.E., Jessup,S.J. and Ramwell,P.N.(1972) Adv.Cyc.Nuc.Res. 1, 479.

Sherline,P.,Lynch,A. and Glinsmann,W.H.(1972) Endocrinology 91, 680.

Shigeta,Y. and Shreeve,W.W.(1966) Am.J.Physiol. 206, 1085.

Shrago,E., Lardy,H.A., Nordlie,R.C. and Foster,D.O. (1963) J.Biol.Chem. 238, 3188.

Stoerk,H.C. and Porter,C.C.(1950) Proc.Soc.Exptl.Biol.Med. 74, 65.

Struck,E., Ashmore,J. and Weiland,O. (1965) Biochem. Z. 343, 107.

Stubbs,M., Kirk,C.J. and Hems,D.A.(1976) Febs Letters, 69, 199.

Sutherland,E.W. and Rall,T.W.(1958) J. Biol.Chem.232, 1077

Thompson,W.J., Williams,R.H. and Ashleman,B.T.(1973).Reference 4 in Thompson,W.J., Williams,R.H and Little,S.A.(1973) Biophys.Acta 302, 329.

Thorn,G.W., Koepf,G.F., Lewis,R.A. and Olsen,E.F. (1940) J.Clin.Invest. 19, 813.

Thorn,G.W., Renold,A.E. and Weingrad,A.I.(1957) Brit.Med.J. 2,1009

Thorp,J.M.(1963) in: The Control of Lipid Metabolism,(Ed.Grant,J.K.) p.163, Academic Press,(New York).

Tilton,M.M., Torralba,G. and Ingle,D.J. (1955) Metabolism 4, 424.

Tolbert,M.E.M. and Fain,J.N.(1974) J.Biol.Chem. 249, 1162.

Tolbert,M.E.M., Butcher,F.R. and Fain,J.N. (1973) J.Biol.Chem. 248, 5686.

Topping,D.L. and Mayes,P.A.(1972) Biochem.J. 126, 295.

Tracht,M.E., Goldstein,M.S. and Ramey, E.R.(1956) Am.J.Physiol. 187, 2.

Uete,T. and Ashmore,J. (1963) J.Biol.Chem. 238, 2906.

Ui,M., Exton,J.H. and Park,C.J.(1973a) J.Biol.Chem. 248, 5350.

Ui,M., Claus,T.H., Exton,J.H. and Park,C.R.(1973b) J.Biol.Chem. 248, 5344.

Vaisler,L., Costiner,E. and Gheorghin,C.(1965) Stud.Cercet.Endocr.16,577.

Van de Werve,G.,Hue,L., and Hers,H.G. (1977) Biochem.J. 162, 135.

Van Lan,V., Yamaguchi,N., Garcia,M.J. Ramey,E.R. and Penbos,J.C. (1974) Endocrinology 94, 671.

Veech,R.L., Eggleston,L.V. and Krebs,H.A.(1969) Biochem.J. 115, 609.

Veneziale,C.M.(1972) Biochemistry 11, 3286.

Volpe,J.J. and Marasa,J.C.(1975) Biochim.Biophys.Acta. 380, 454.

Walsh,D.A., Perkins,J.P. and Krebs,E.G.(1968) J.Biol.Chem. 243, 259.

Weber,G., Singhal,R.L., Stamm,N.B., Fisher,E.A. and Mentendiek,M.A. (1964) Ad.Enz.Reg. 2, 1.

Welt,I. and Wilhemi,A.E. (1950) Yale J. Biol.Med. 23, 99.

Wheldrake,J.F.(1972) Biochim.Biophys.Acta. 260, 583.

Whitton,P.D. and Hems,D.A.(1976) Biochem.J. 156, 585.

Wilcox,H.C., Dishman,G. and Heimberg,M.(1968) J.Biol.Chem. 243, 666.

Williamson,D.H. and Mellanby,J. (1963) in: Methods of Enzymatic Analysis, p.454, (Ed.Bergmeyer H.V.), Academic Press,(New York).

Williamson,D.H., Lund,P. and Krebs,H.A.(1967) Biochem.J. 103, 514.

Williamson,J.R.(1966) Biochem.J. 101, 11C.

Willmer,J.S. and Foster,T.S.(1960) Can.J. Bioch.Physiol. 38, 1387.

Windmueller,H.G. and Spaeth,A.E.(1966) J.Biol.Chem. 241, 2891.

Winternitz,W.W., Dintzis,R. and Long,C.N.(1957) Endocrinology 61, 724.

Wong,F.C. (1950) Nature 165, 277.

Wood,R. and Harlow,R.D.(1969) Arch.Biochem.Biophys. 131,495.

Zahlten,R.N., Kneer,N.M.,Stratman,F.W. and Lardy,H.A.(1974) Arch. Biochem.Biophys. 161, 528.