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THE EFFECTS OF FREE FATTY ACIDS AND PHENFORMIN
ON HEPATIC GLUCOSE SYNTHESIS AND RELEASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the
Degree Doctor of Philosophy in the Graduate School of
The Ohio State University

By

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* * * * * * *

The Ohio State University
1966

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CHAPTER I

PROBLEM STATEMENT

Glucose and fatty acids comprise the major fuels that are oxidized to supply energy for the body. Fatty acids are stored mainly as muscle and adipose tissue triglycerides, whereas glucose is stored as muscle and liver glycogen. However, only the liver and adipose tissue effectively contribute these fuels to the circulation. Randle et al. (1) have recently proposed that glucose and fatty acid metabolism in muscle and adipose tissue form a self-regulating "glucose fatty acid cycle" which is fundamental to the control of blood glucose and fatty acid concentrations. According to Randle and his co-workers, free fatty acids inhibit the uptake of glucose by muscle tissue, thus tending to elevate blood glucose. The elevation in blood glucose would, in turn, tend to diminish free fatty acid release from adipose tissue by providing the glycerol-phosphate necessary for reesterification of these fatty acids. Increased blood glucose levels would also trigger the release of insulin, further enhancing reesterification and suppressing lipolysis. According to these authors, the glucose fatty acid cycle provides a primitive mechanism which would tend to maintain blood glucose concentration independently of hormonal control.

The proposed glucose fatty acid cycle does not include the liver, which is the major source of blood glucose. When glucose
levels become elevated following a carbohydrate-rich meal, the liver rapidly takes up glucose, storing some of it as liver glycogen and converting much of the rest to lipid. In the post-absorptive state the blood glucose begins to fall and the liver then slowly breaks down its glycogen stores, releasing glucose into the bloodstream.

The liver also is capable of synthesizing glucose from non-carbohydrate precursors such as amino acids, lactic acid, and glycerol, which it can then release into the blood or store as glycogen. The kidney is the only other organ capable of gluconeogenesis (synthesizing glucose from non-carbohydrate precursors); it contributes approximately ten to fifteen per cent of the circulating glucose in man (2).

Regulation of blood glucose concentration is extremely important to the animal. Erythrocytes and nervous tissue rely almost solely on blood glucose as a source of energy, since they are unable to oxidize fatty acids (2). Severe hypoglycemia frequently causes the animal to lose consciousness because the brain receives insufficient glucose. Hyperglycemia, on the other hand, results in a wasteful loss of glucose to the organism since the kidney can reabsorb only a certain amount of glucose from the glomerular filtrate. When blood sugar levels are elevated, glucose is often spilled into the urine. Normally blood glucose concentrations remain fairly constant. Even after a carbohydrate-rich meal blood glucose rarely exceeds 130 mg.% and seldom falls below 50 mg.% even during prolonged fasting (3).
The glucose fatty acid cycle proposed by Randle et al. stressed the effects of blood glucose on fatty acid release by adipose tissue. We have tried to extend the concept of a reciprocal glucose fatty acid relationship by investigating the effects of fatty acids on glucose release by the liver.

Several hormones tend to elevate blood glucose concentration. The glucocorticoids increase blood glucose by enhancing gluconeogenesis (4). Glucagon and epinephrine enhance glycogenolysis and are also capable of increasing free fatty acid concentrations by stimulating lipolysis (5,6). Conversely, insulin has a hypoglycemic effect, attributable to its inhibition of gluconeogenesis and enhancement of peripheral utilization of glucose (7). Insulin also inhibits the release of free fatty acids from adipose tissue (8).

Our understanding of the mechanisms by which these hormones act is, by no means, complete. For example, there is disagreement about whether insulin has an immediate and direct effect on glucose output by the liver (9).

Insufficient insulin production by the pancreas results in the disease diabetes mellitus, which is characterized by severe hyperglycemia. Patients suffering from this disease usually respond well to daily injections of insulin. There has been an extensive search for compounds which can be taken orally and which will correct the metabolic defects in diabetes. Such sulfonylureas as tolbutamide have proved to be effective oral hypoglycemic drugs; however, their use is restricted to those patients possessing functional pancreatic
islet tissue (10). The biguanides have also met with limited success as oral hypoglycemic agents. These compounds apparently can act in the absence of functional islet tissue; however, their mechanism of action has not been clearly elucidated (11). We have investigated the mechanism of action of the biguanides, with special emphasis on their hepatic effects.
CHAPTER II
HISTORICAL REVIEW

Control of blood glucose

Almost three centuries ago (1674) Willis noted that diabetic urine possessed a sweet taste (12). This was the first mention of glycosuria in the European literature on diabetes. However, ancient Japanese, Chinese and Hindu writings indicated that these people were aware of a disorder associated with sweet urine (13). Around 1000 A.D. the Arab physician Avicenna gave a complete description of the disease diabetes mellitus and remarked that the urine of diabetic patients possessed a honey-like substance (13). In 1788, Cawley isolated glucose from the urine of persons afflicted with diabetes mellitus (12,13). Glucose was not regarded as a normal constituent of blood until 1846, when Magendie demonstrated its presence in the blood of normal animals during the digestive absorption of starches (14).

Claude Bernard was the first to appreciate the importance of glucose to the economy of the animal and to suggest that the liver played a role in its production and storage (12,14). He demonstrated that the livers of dogs which had been fed nothing but meat for a long period of time contained both glucose and a substance convertible to glucose in the presence of saliva. Because of its similarity to starch, he described this compound as animal starch, and since it
yielded glucose in the presence of "ferments" he named it glycogen. Bernard argued that since these meat-fed dogs derived no carbohydrate from their diets, this glucose had to be manufactured by the animal and he further postulated that the synthesis occurred in the liver. This hypothesis was supported by the work of Moleschott when he demonstrated that blood sugar disappeared from frogs whose livers had been removed (14). Many years later, in 1922, Mann and Magrath (15) performed similar experiments on dogs. They reported that total removal of the liver invariably led to a progressive decrease in the blood glucose concentration, followed by coma, and then convulsions terminating in death. The administration of glucose temporarily prolonged the lives of these animals but they eventually succumbed due to factors unrelated to blood glucose concentration.

The discovery that the glycoside phlorhizin caused an animal's carbohydrate reserves to be drained off into the urine greatly facilitated the study of glucose production by the animal. Administered glucose could be recovered almost quantitatively in the urine of phlorhizin diabetic animals and the administration of any substance giving rise to blood glucose caused an increased excretion of glucose (16). Using phlorhizin diabetic animals, Lusk et al. (17) demonstrated that the ingestion of meat was followed by greatly increased glucose elimination. They calculated that approximately 58% of dietary protein was converted to glycogen and then to glucose (18). However, Folin (19) advanced the idea that protein was degraded completely to amino acids during digestion and that these
amino acids were absorbed and converted to glucose. He stated that in normal animals when ingested protein was in excess of that needed, the body (probably the liver) eliminated the nitrogenous part as urea "so as to get the use of the carbonaceous part."

The next several years witnessed the systematic investigation of the gluconeogenic potential of various substances. Propyl alcohol, propionic acid, lactate, fructose, galactose, succinate, malate, glycerol, and the amino acids glycine, alanine, serine, cysteine, aspartic acid, glutamic acid, ornithine, proline, and arginine were shown to be gluconeogenic (20,21,22,23). However, the amino acids leucine, phenylalanine and tyrosine yielded ketone bodies (beta-hydroxybutyric acid, acetoacetic acid, and acetone) rather than glucose (24). Thus, amino acids were classified according to whether they were gluconeogenic or ketogenic.

It was generally believed that gluconeogenesis was the reverse of Embden-Meyerhoff glycolysis. However, Krebs pointed out that barriers occurred at several steps which effectively prevented this reversal. Barriers occurred between pyruvate and phosphoenolpyruvate, between fructose-1,6-diphosphate and fructose-6-phosphate, and between glucose-6-phosphate and glucose (2). In 1949 de Duve et al. reported the discovery of a specific glucose-6-phosphatase (25). The activity of this enzyme could be detected only in the liver, kidney and intestines. This prompted the suggestion that glucose-6-phosphatase was the "main gateway" by which glucose entered the bloodstream. Fructose-1,6-diphosphatase was discovered in 1943 by
Gomori (26) and in 1956 Mokrash et al. noted that the enzyme was present in large amounts in the liver (27). In 1964, Underwood and Newsholme investigated the properties of fructose-1,6-diphosphatase in an attempt to determine how its metabolic activity was controlled. Adenosine monophosphate and fructose-1,6-diphosphate were found to inhibit fructose-1,6-diphosphatase activity; thus the authors suggested that these two compounds were regulatory effectors of the enzyme. They emphasized the importance of this regulation as a possible mechanism for the control of gluconeogenesis (28).

The conversion of pyruvate to phosphoenolpyruvate was shown to proceed in two steps. In 1954, Utter and Kurahashi discovered an enzyme in pigeon liver which converted oxaloacetate plus GTP to phosphoenolpyruvate and GDP (29). It was believed that this enzyme, phosphoenolpyruvate carboxykinase, was important in gluconeogenesis. However, at that time, the only known path for the conversion of pyruvate to oxaloacetate involved malic enzyme; pyruvate was first converted to malate by malic enzyme; the malate was then oxidized to oxaloacetate by malic dehydrogenase (30). It was thus suggested that pyruvate was converted to phosphoenolpyruvate through the action of malic enzyme, malic dehydrogenase, and then phosphoenolpyruvate carboxykinase.

However, in 1960, Utter and Keech discovered pyruvic carboxylase which catalyzed the direct conversion of pyruvate to oxaloacetate (31):

\[ \text{Pyruvate} + \text{CO}_2 + \text{ATP} \xrightarrow{\text{Pyruvic Carboxylase}} \text{Oxaloacetate} + \text{ADP} + \text{Pi} \]
Since this enzyme increased in amount in the livers of fasted rats, whereas malic enzyme did not, it was concluded that pyruvate entered the gluconeogenic pathway by being carboxylated to oxaloacetate (32).

Thus the energy barriers which prevented the reversal of glycolysis were shown to be circumvented by reactions unique to gluconeogenesis, and the enzymes involved were found predominantly in the liver and kidney, the two organs primarily responsible for supplying blood glucose.

Although it was established that amino acids were gluconeogenic, there was considerable debate as to whether or not fat could give rise to blood glucose. Pflüger investigated this problem by feeding dogs nothing but fat for an extended period of time; when these animals were sacrificed he found very little liver glycogen and therefore concluded that fat was not a source of carbohydrate (33). As early as 1902, Cremer emphasized that glycerol and the glycerol moiety of fat could be converted to glucose (34). It was finally concluded that although fatty acids themselves (with the exception of those containing an odd number of carbon atoms and thus giving rise to propionic acid in their metabolism) could not be converted to glucose, the glycerol portion of the triglyceride molecule was strongly gluconeogenic.

However, evidence suggesting the conversion of fatty acids to glucose continued to appear in the literature. In 1938 Weil-Malherbe observed increased glucose production by kidney slices in the presence of acetoacetate (35). Krebs helped clarify this situation
when, in 1963, he demonstrated that acetoacetate stimulated the conversion of non-carbohydrate precursors to glucose in rat kidney slices without themselves being incorporated into glucose. He explained this phenomenon by recalling that pyruvic carboxylase required acetyl CoA for its activity and suggested that acetoacetate supplied this acetyl CoA in the kidney (36).

Although fat could not be converted to glucose, the reverse reaction, the conversion of glucose to fat has been known for many years. Early workers noted that the force feeding of carbohydrates resulted in an accumulation of fat greatly in excess of that found in the diet (37). Recent isotopic studies have confirmed the fact that glucose is readily converted to fatty acids (38). Most of this conversion was shown to take place in the adipose tissue rather than in the liver as was previously thought (39). It was estimated that some 30% of dietary carbohydrate is converted to fatty acids before being further metabolized and that 90% of stored glucose carbon is stored as fat (40). It was once widely held that carbohydrates were the most important fuels for the body and that lipids contributed only after being converted to ketone bodies by the liver. However, recent evidence suggested that the oxidation of fatty acids accounts for 50 to 70% of the oxygen consumed by the animal (41), and that plasma free fatty acids are the immediately utilizable source of energy for oxidation in muscle tissue (42).

The free fatty acid fraction of plasma was largely neglected for many years since it accounted for only 5% or less of the total
plasma lipids (43). In 1956 Dole (44), Gordon (45) and Laurell (46) discovered that plasma free fatty acid concentrations changed rapidly during carbohydrate administration, starvation, and after the administration of epinephrine. That same year Havel and Fredrickson (47) demonstrated that free fatty acids of dog plasma were turned over very rapidly, having a half-time of two to four minutes. Thus, although the free fatty acid concentration of plasma was shown to be only about 15 mg%, its rapid turnover indicated that it was metabolically important and could account for a large percentage of the fat oxidized by the body.

Plasma free fatty acids are derived almost exclusively from the hydrolysis of adipose tissue triglycerides. The release of free fatty acids from adipose tissue was shown to be extremely susceptible to hormonal influence. Epinephrine, norepinephrine, growth hormone, and glucagon have all been shown to stimulate the release of fatty acids by activating lipase, whereas insulin has been shown to inhibit the release of fatty acids from adipose tissue (8,49). Wood (50) suggested that insulin promoted the reesterification of fatty acids by making alpha-glycerol phosphate available through enhanced glucose utilization. Jungas and Ball (51) suggested that insulin inhibited fatty acid release by inhibiting lipase activity as well.

Most of the free fatty acids mobilized from adipose tissue were shown to be taken up by liver and muscle. Soon after the injection of labeled albumin-bound palmitate-1$^{14}$C, Bragdon and
Gordon (52) were able to recover one-third of this label in muscle and one-third in liver. The amount of free fatty acids extracted from the blood in vivo was shown by Spitzer and McElroy (53) to be proportional to the free fatty acid concentration in the portal blood. Havel (54) demonstrated that these fatty acids were rapidly esterified and Nestel and Steinberg (55) found that perfusing isolated livers with high concentrations of free fatty acids resulted in a 25 to 60% increase in liver triglycerides.

Some of these fatty acids were oxidized rather than being converted to triglycerides. Using liver homogenates, Ontko (56) demonstrated that when fatty acid concentrations were low, oxidation resulted solely in the production of CO$_2$. However, at higher fatty acid concentrations the oxidation of fatty acids resulted in the production of acetoacetate. Ontko suggested that the production of acetyl CoA exceeded the capacity of the Krebs cycle to further oxidize acetyl CoA. That the overproduction of acetyl CoA was associated with ketogenesis was supported by the work of Wieland et al. (57). These authors reported that acetyl CoA levels were increased two to three fold in the livers of ketogenic alloxan diabetic and fat fed animals.

Acetyl CoA molecules not oxidized via the Krebs cycle were shown to condense to form acetoacetyl CoA. Lynen et al. (58) presented evidence that acetoacetate production from acetoacetyl CoA involved a two step sequence of reactions. Acetoacetyl CoA and acetyl CoA condensed to form beta-hydroxy-beta-methylglutaryl CoA
and free co-enzyme A. The condensation product was then cleaved forming acetyl CoA and free acetoacetate. The direct deacylation of acetoacetyl CoA was demonstrated by other workers (59). The relative significance of the two pathways for the production of acetoacetate has not been determined.

Many years ago it was discovered that various endocrine organs profoundly affect blood glucose concentration. This was clearly demonstrated in 1889 when von Mering and Minkowski produced diabetes mellitus in dogs by removing their pancreases (60). They reported that total pancreatectomy produced a disease state similar to diabetes in man. These dogs exhibited polyuria, ketonuria, glucosuria, and hyperglycemia. Insulin, the active principle from the pancreas, was isolated by Banting and Best in 1921 (61). Insulin had a marked hypoglycemic effect when injected into an animal and was effective in alleviating the symptoms of diabetes.

However, most insulin injections caused a paradoxical initial hyperglycemia which was finally attributed to a contaminant found in insulin preparations (62). The contaminant turned out to be glucagon, another pancreatic hormone, which was shown to stimulate hepatic glycogenolysis by activating liver phosphorylase (63).

It had been known for many years that epinephrine, produced by the adrenal medulla, also caused glycogen breakdown. Rall and Sutherland (64) studied the mechanism by which epinephrine caused glycogenolysis. They demonstrated that liver phosphorylase was present in both an active and an inactive form, the rate of glycogen
breakdown being determined by the amount of active phosphorylase present. Inactive phosphorylase, or dephosphophorylase, was converted to active phosphorylase by dephosphophorylase kinase and ATP. The kinase required 3'5'-cyclic AMP for activation; cyclic AMP was produced from ATP under the influence of the enzyme adenyl cyclase. Epinephrine and glucagon were shown to activate phosphorylase by stimulating the cyclase reaction.

The apparent antagonistic effects of insulin and epinephrine led numerous early workers to postulate that blood glucose was regulated by the balanced secretion of these two hormones (65). Glucose administration caused a secretion of insulin which tended to lower blood sugar; hypoglycemia, on the other hand, caused a discharge of adrenalin which tended to raise blood sugar. According to Somogyi (66), "Whenever the glycemic level is changed from the normal fasting level a compensatory change in the regulatory mechanism ensues to restore the original level." This simple picture of blood glucose regulation was later complicated by the discovery that glucagon and the adrenal cortical hormones also influence blood glucose concentration to a marked degree.

In 1936 Buell et al. (67) showed that adrenalectomized animals deposited very little liver glycogen following the ingestion of lactic acid. This effect was reversed when the animals were treated with adrenal cortical extracts. Working with adrenalectomized rats and mice, Long et al. (68) found that these animals maintained normal carbohydrate levels when fed. However, when these animals
were fasted, their blood glucose declined more rapidly than did the blood glucose of fasted normal animals. These animals also excreted less urinary nitrogen than did normal animals. The administration of adrenal cortical extracts to adrenalectomized animals resulted in large increases in liver glycogen and a slight hyperglycemia. Urinary nitrogen increased to such an extent that Long et al. (68) suggested that protein catabolism was the source of the newly formed carbohydrate. That same year Lewis et al. (69) reported that adrenalectomized animals exhibited impaired ability to form glucose from lactic acid, pyruvic acid or alanine, increased utilization of available glucose, and decreased ketonuria during fasting after the administration of phlorhizin. The effect of the adrenal cortical hormones on gluconeogenesis was shown to be due, in part, to their effects on the key gluconeogenic enzymes. In 1963, Henning (70) reported that cortisol administration increased the levels of pyruvic carboxylase in rat liver. Weber and Singhal (71) demonstrated that glucose-6-phosphatase and fructose-1,6-diphosphatase were increased by over 200% following daily injections of cortisol. Weber (72) has postulated that the glucocorticoids induce the synthesis of the gluconeogenic enzymes and that insulin inhibits this synthesis. However, Lardy (73) has disagreed with this point of view. He suggested that the main function of the glucocorticoids was to mobilize protein for conversion to glucose.
Hypoglycemic guanidine derivatives

The chance observation by Watanabe (74) in 1918, that guanidine caused profound hypoglycemia in rabbits, provided the impetus for an intensive search for oral hypoglycemic agents which could be used in the treatment of diabetes. Unfortunately, guanidine itself was entirely too toxic to be considered as a potential therapeutic agent, but in 1926, Frank et al. (75) synthesized the guanidine derivative decamethylenediguanidine (synthalin) which exhibited not only far less toxicity than guanidine, but increased hypoglycemic activity as well.

Synthalin was found to cause an initial hyperglycemia (as did guanidine itself) in normal fed rabbits (76). Large doses (10 mg./kg.) produced hypoglycemic convulsions after four or five hours which could be relieved by the administration of glucose, but unlike the convulsions produced by insulin, they were not relieved by the injection of epinephrine (76). This was due partly to the fact that synthalin depleted the liver's glycogen reserves. In diabetic animals synthalin abolished hyperglycemia and glycosuria but, according to Hesse and Taubman (77) the diabetic animals did not "perk up" as when given insulin. Kaufmann-Cosla and Vasilco (78) stated that the disappearance of glycosuria in the diabetic animal was only apparent since the urine of these animals contained such large quantities of organic substances that the urinary excretion of carbon compounds was unchanged. They felt that synthalin did not alleviate the impaired metabolism of the diabetic.
Nevertheless, Frank and his associates (79) experienced some success in the clinical treatment of mild diabetics with synthalin, and this compound was used for several years as a therapeutic agent. Reports of the occurrence of liver and kidney damage in some patients treated with synthalin terminated its use as an oral hypoglycemic agent (80).

The synthesis of N'-beta-phenethylformamidinyliminoura HCl (DBI or phenformin) by Ungar and his group (81) in 1957 caused renewed interest in guanidine derivatives as possible oral hypoglycemic drugs. It was reported that, unlike synthalin, DBI produced no pathological changes in the kidneys, liver, spleen or heart of animals which expired as a result of the hypoglycemia produced by this compound.

Clinical trials showed that DBI was effective in lowering the blood glucose concentrations in 89% of those diabetics tested, and that it was effective in lowering the insulin requirements of juvenile-onset diabetics (82). Unfortunately, DBI frequently caused gastrointestinal distress such as nausea, vomiting, and diarrhea, but these symptoms were believed to be unrelated to the hypoglycemic activity of the drug since they occurred as frequently in those patients who experienced no blood sugar-lowering as in those who did (82). The possible therapeutic value of this drug led to numerous investigations dealing with its mechanism of action.

Tybergh Hein and Williams (83) demonstrated that in guinea pigs, subcutaneously administered DBI caused hypoglycemia and depletion of
liver glycogen. Further studies showed that the addition of DBI to guinea pig liver slices resulted in increased lactate formation and glycogen depletion without a change in net glucose output. Incubating isolated hemi-diaphragms with DBI increased glucose uptake and lactic acid production by this tissue while decreasing glycogen formation and oxygen utilization. These authors concluded that the hypoglycemia produced by DBI could be attributed to increased anaerobic glycolysis caused by tissue anoxia. However, Nielsen et al. (84) demonstrated diminished hepatic glucose output in guinea pigs treated with 20 mg./kg. DBI and Williams et al. (85) presented evidence which suggested that DBI inhibited gluconeogenesis. According to Steiner and Williams (86), the presence of tissue anoxia could also explain the decrease in gluconeogenesis since active oxidation is required for the deamination of amino acids and for the reversal of the Embden-Meyerhof pathway in the conversion of pyruvic acid to glucose.

Experiments with human subjects showed that DBI produced blood sugar lowering only in diabetic patients; it had little or no effect on the blood glucose levels of normal volunteers (87). Diabetic patients exhibited increased blood and urine levels of both lactate and pyruvate in addition to diminished blood glucose concentration. Madison and Unger (88) postulated that DBI decreased glucose output by the liver of diabetics since they could demonstrate no enhancement of peripheral glucose utilization by examining femoral arterial-venous glucose differences following the administration of this drug. On the other hand, Tranquada et al. (89)
concluded that DBI did not diminish hepatic glucose output since they could demonstrate no such effect using the technique of hepatic vein catheterization of diabetic patients receiving DBI and exhibiting a hypoglycemic response. However, the data obtained by these workers appeared to be extremely variable and difficult to interpret. The accumulation of labeled DBI in the stomach and liver following either oral or parenteral administration led Wick et al. (90) to suggest that DBI acts primarily on the liver. They further postulated that the liver produces increased quantities of lactate and pyruvate which can then be used as a source of energy for peripheral tissue since the uptake of these compounds is not insulin-dependent.

The effects caused by DBI, i.e., decreased oxygen consumption, increased levels of lactate, and increased glycogenolysis suggested that it was a respiratory inhibitor. Wick et al. (91) were able to demonstrate that DBI inhibited the oxidation of succinate in rat liver homogenates. Hollunger (92) demonstrated the inhibition of mitochondrial oxidation by guanidine and reported that this inhibition was reversed when oxidation became uncoupled from phosphorylation. He concluded that guanidine inhibited the mechanism by which ADP reacted with the respiratory chain to form ATP. The high energy bonds generated during electron transport had to be removed before electron transport, and therefore oxygen consumption could continue. By interfering with the reaction of the high energy containing respiratory chain with ADP, guanidine effectively blocked
oxygen consumption. When oxidative phosphorylation became uncoupled these high energy bonds were dissipated so that oxygen consumption could proceed without the formation of ATP; in this case guanidine no longer inhibited oxygen consumption. Kruger et al. (93) were able to demonstrate a similar phenomenon using DBI itself rather than guanidine.

Liver perfusion

The perfusion of surviving organs has proved to be a valuable tool for investigating the metabolism of individual organs isolated from the influences of the rest of the body. This technique has enabled investigators to control such variables as hormone and metabolite levels without disrupting the structural and functional integrity of the organ.

A method for the perfusion of surviving muscle tissue was first described by Ludwig and Schmidt in 1869 (94). Several years later, in 1875, Luchinger (95) described experiments with the isolated perfused liver. He reported that perfusing a liver with blood containing 2% dextrose resulted in an increased concentration of liver glycogen. A perfusion apparatus not unlike those in use today was constructed by Brodie in 1903 (96). That same year, using Brodie's perfusion apparatus, Grube (97) was able to demonstrate an increase in both glycogen and glucose in the livers of cats which had been perfused with defibrinated whole blood for two hours. In 1907 he reported that glycerol, glucose, fructose, and galactose
were converted to glycogen in the isolated perfused turtle liver (98). However, he was unable to demonstrate the conversion of amino acids to glucose. Pechstein (99), Masing (100), and Frohlich and Pollak (101) all reported that the addition of epinephrine to the blood perfusing an isolated liver resulted in an immediate rise in perfusate glucose. Insulin was without effect on glycogen deposition or glycogenolysis in the isolated perfused turtle liver (102).

Around the turn of the century perfusion techniques were in vogue, and interest in these procedures continued until they were superseded by the simpler liver slice technique introduced in 1923. From 1923 until 1951 only a few reports concerning liver perfusions appeared in the literature.

In 1926, Burn and Marks (103) investigated carbohydrate production in isolated perfused livers from fat-fed dogs. They noted that during perfusion with whole blood, even in the absence of added substrate, there was an increase in total carbohydrate (glucose plus glycogen); this carbohydrate production was unaffected by the addition of insulin or thyroid extract. They also reported a large production of ketone bodies by the isolated liver.

Lundsgaard et al. (104) made several improvements on existing perfusion techniques in 1936. They preperfused the livers in situ in order to minimize the period of anoxia which previously attended the surgical procedure, and they oxygenated the perfusate with 95% oxygen and 5% carbon dioxide rather than with air or pure oxygen. These authors reported that cat livers did not utilize glucose or
produce lactate, but they did synthesize glycogen from lactate. Rabbit livers were shown to be different from those of cats in that they did utilize glucose and produce lactic acid. These workers could demonstrate no effect of insulin on isolated livers from cats or rabbits.

Early in the 1950's there was renewed interest in liver perfusion. By this time it was evident that results obtained with liver slices did not always agree with results from in vivo experiments. Liver slices did not synthesize glycogen when exposed to normal glucose concentrations. The effects of epinephrine and glucagon on liver slices were not always as pronounced as in the liver of the whole animal (105). Liver slices were composed of a great many ruptured and dying cells. Perfused livers, on the other hand, more closely resembled the liver in vivo since the structural and functional integrity was maintained.

Several new perfusion techniques appeared in the early 1950's. In 1951 Brauer et al. (106) devised a technique which enabled them to perfuse rat livers up to twenty-five hours. At the end of this time these livers appeared normal histologically and were capable of excreting the dye BSP. This technique did not gain wide acceptance since it was extremely complicated and required a sterile perfusion apparatus and sterile technique throughout the operative procedure. A far simpler method was introduced by Miller et al. (105) that same year. It was similar in principle to those described by various early workers. However, the inclusion of
filters eliminated problems caused by clot formation and an improved oxygenation system prevented anoxia. These workers reported that their preparation was viable for up to seven hours and was capable of synthesizing plasma proteins. Miller and his co-workers concluded that liver perfusion afforded a method for studying liver metabolism in vitro under conditions simulating those in the intact animal. They stressed the fact that in the perfused liver, cell membranes and exchange surfaces were left intact. Unlike liver slices, they were untraumatized by handling, slicing, compression or rupture and uncompromised by relying upon diffusion through a passive slice to supply oxygen and substrate and to remove products and wastes.
CHAPTER III
MATERIALS AND METHODS

Perfusion technique

The perfusion technique and apparatus were adapted from those of Miller (105). Male rats or guinea pigs, anesthetized with diethyl ether, were employed as liver donors. The abdominal cavity was opened with a midline incision and the viscera were displaced so that the portal vein and common bile duct were exposed. The rat bile duct was cannulated with a three inch length of P.E. 50 polyethylene tubing which was then tied securely in place. In the case of the guinea pig the gall bladder was first removed and the cystic duct was tied off, the common bile duct then being cannulated by a three inch length of P.E. 205 polyethylene tubing. The portal vein and the inferior vena cava cephalad to the right renal vein were then ligated. The portal vein was entered with a seventeen gauge needle encased by a two cm. length of P.E. 205 polyethylene tubing, the needle itself being attached to a reservoir containing oxygenated Krebs-Ringer-bicarbonate buffer. Pre-perfusion with this buffer was begun immediately, the inferior vena cava caudad to the right renal vein was divided, and the ligature around the vena cava was tied securely. The liver was rapidly excised and transferred to the perfusion apparatus. The entire surgical procedure required from
five to ten minutes and the period of ischemia lasted no more than a few seconds. Viability of the liver was judged by general appearance, bile production (0.5-0.6 ml./hour by the rat liver and 3.0-6.0 ml./hour by the guinea pig liver), and perfusion rate (generally between 30-55 ml./min. at a 12-14 cm. hydrostatic head). The liver was maintained at a constant temperature of 28-30°C during perfusion and the perfusate was oxygenated with a 95% oxygen-5% carbon dioxide mixture.

In some experiments the livers were perfused with out-dated human serum albumin which was generously supplied by the American Red Cross. This albumin was found to contain extremely large concentrations of free fatty acids (3000 μEq./l. of a 5% solution) and therefore a procedure was devised for removing the majority of these fatty acids without denaturing the albumin. Approximately 250 ml. of a 10% solution of albumin in Krebs-Ringer-bicarbonate buffer were perfused through a liver for six to eight hours; the liver readily oxidized these fatty acids and produced large quantities of ketone bodies. The albumin was then passed through a Seitz filter to remove any particulate matter (such as erythrocytes, denatured protein, and bacteria) and dialyzed against Krebs buffer for 24 hours. The albumin solution was diluted with buffer so that the final protein concentration was approximately 5%. This constituted the 'low fat' perfusate which contained 300-600 μEq./l. free fatty acids. 'High fat' albumin was prepared by adding concentrated aqueous sodium oleate to the 'low fat' albumin. This
solution was warmed to approximately 40°C to facilitate the binding of the fatty acids to the albumin, and then filtered to remove traces of insoluble calcium soaps.

In experiments where free fatty acids were added during the course of the perfusion, they were added as a concentrated albumin-fatty acid complex. This complex was prepared by mixing warm (40°C) aqueous sodium oleate with warm 25% salt-poor albumin; this resulted in the formation of a clear 5% albumin solution containing 200 μEq./ml. free fatty acids. Fatty acids were added in this manner to avoid the initial formation of insoluble calcium soaps which would interfere with the perfusion.

The perfusion apparatus consisted of a one-liter rotating flask in which the perfusate was oxygenated, a pump, an upper reservoir which was employed to maintain a constant hydrostatic head of 12-14 cm. and a chamber which supported the liver. The perfusate was pumped from the flask through a blood filter into the upper reservoir. From there it flowed through the liver by gravity and was returned to the rotating flask. A stopcock between the liver and flask permitted easy sampling of the perfusate. The perfusion apparatus is illustrated in Figure 1. Frequently a parallel perfusion system was employed in which two flasks, two pumps, and two upper reservoirs were used. Three-way stopcocks immediately before and after the liver enabled one to change completely from one perfusate to another without interrupting the perfusion (Figure 2).
FIGURE 2
PARALLEL PERFUSION APPARATUS
Sampling

In all experiments the initial volume of perfusate was 100 ml. The AutoAnalyzer withdrew 9.6 ml. of perfusate each hour and 10 ml. samples were taken each half hour. The same sampling procedure was used in all experiments. This permitted comparisons to be made between metabolite concentrations in the perfusate.

Glucose

A sample tube from the AutoAnalyzer was placed in the rotating flask; this allowed the continuous monitoring of the perfusate glucose concentration. Glucose was determined by measuring the decrease in absorbency at 430 μm of an alkaline potassium ferricyanide solution. Glucose reacts with the yellow ferricyanide solution reducing it to colorless ferrocyanide.

Free fatty acids

Free fatty acids were determined by extracting a sample of perfusate with acidified isopropyl alcohol-heptane mixture and titrating with sodium hydroxide according to the method of Dole (44).

Lactate and pyruvate

Lactate and pyruvate were determined enzymatically using commercially available kits (C.F. Boehringer & Soehne, Mannheim). Pyruvate was determined by converting it to lactate and the change in absorbency at 340 μm, a measure of the change in NADH concentration, was recorded. This was proportional to the pyruvate concentration.
\[
\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{Lactic Dehydrogenase}} \text{Lactate} + \text{NAD}^+
\]

Lactate was determined by the reverse reaction which was driven to completion by the inclusion of hydrazine which reacted with the pyruvate as it was produced.

**Glycerol**

Glycerol was determined enzymatically using the coupled reaction sequence devised by Garland and Randle (107).

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol-1-Phosphate} + \text{ADP}
\]

\[
\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{Pyruvic Kinase}} \text{Pyruvate} + \text{ATP}
\]

\[
\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{Lactic Dehydrogenase}} \text{Lactate} + \text{NAD}^+
\]

The change in optical density at 340 \(\mu\) was proportional to the glycerol concentration.

**Total ketones and acetoacetate**

Acetoacetic acid and total ketones were determined by a modification of the method of Lyon and Bloom (108). For the determination of acetoacetate plus acetone, a protein free filtrate was prepared by combining equal volumes of 10% zinc sulfate, 0.5N sodium hydroxide and perfusate, and centrifuging for ten minutes. One ml. of supernatant solution and one ml. 4.0N sulfuric acid were added to a Thünïberg tube. This was placed in a 98°C water bath and the stopper, which contained sodium hydroxide and furfural was immersed in a cold water bath. The heat and acid decomposed acetoacetate to acetone, and the acetone (both preformed and derived
from acetoacetate) distilled into the stopper and was trapped as difurfurylidene-acetone. This compound reacted with strong acid to produce a red color which was measured at 530 nm. Total ketone bodies were determined in the same way except that potassium dichromate was added to the Thünberg tube to oxidize the beta-hydroxybutyrate.

**Preparation of glucose phenylosazones**

In some cases gluconeogenesis was studied using radioactive substrates and the labeled glucose produced was isolated as the phenylosazone. Glucose phenylosazones were prepared by heating for one hour in a boiling water bath, 3 ml. protein free filtrate, 0.1 ml. aniline, 0.5 ml. phenylhydrazine, 50 mg. carrier glucose, and 1.0 ml. glacial acetic acid. The resultant osazone crystals were washed with distilled water and dilute hydrochloric acid, recrystallized from hot ethanol, and washed with hydrochloric acid, water, and diethyl ether. A weighed sample (approximately 20 mg.) of the glucose phenylosazone crystals were wrapped in black combustion paper and placed in an oxygen filled Schoniger flask. They were ignited by an infrared light and the CO$_2$ produced was trapped in hyamine and counted in a Tri-Carb liquid scintillation counter. Corrections for quenching were made by employing toluene-$^{14}$C as an internal standard.
Preparation of liver samples

When tissue levels of metabolites, phosphorylase, and DBI were determined, the liver (or liver biopsy) was clamped between two aluminum blocks which had been cooled in liquid nitrogen. Homogenization was accomplished by grinding the liver with liquid nitrogen using a nitrogen cooled mortar and pestle. The frozen powder was then added to a tared tube containing the appropriate solution and weighed.

Phosphorylase activity

Liver phosphorylase determinations were based on the method of Illingsworth and Cori (109).

Glucose-1-phosphate + Glycogen \(\overset{\text{Phosphorylase}}{\rightarrow}\) Glycogen-glucose + Pi

Frozen liver powder was added to pH 6.1 buffer containing 0.04M glycerol-1-phosphate, 0.03M cysteine, 0.005M EDTA, and 0.1M NaF, mixed thoroughly, and centrifuged at 10,000 xg for ten minutes. Enzyme activity was determined by incubating at 30°C for ten minutes, 0.5 ml. of the extract with 0.5 ml. pH 6.1 substrate which contained 0.032M glucose-1-phosphate and 2% glycogen. The reaction was terminated by the addition of 5 ml. cold trichloroacetic acid. Phosphorylase activity was expressed as \(\mu\)M Pi liberated per gram of tissue per minute.

Adenosine-5'-triphosphate

Adenosine-5'-triphosphate was determined enzymatically on perchloric acid extracts of the frozen liver powder using commercially
available ATP kits (C.F. Boehringer and Soehne, Mannheim). The
determination used the following coupled reaction sequence:

\[
3\text{-Phosphoglycerate} + \text{ATP} \xrightarrow{\text{Phosphoglycerate Kinase}} \text{ADP} + 1,3\text{-di-phosphoglycerate}
\]

\[
1,3\text{-Diphosphoglycerate} + \text{NADH} + H^+ \xrightarrow{\text{Dehydrogenase}} \text{Glyceraldehyde Phosphate}
\]

The glyceroldehyde-3-phosphate was trapped with hydrazine in order to
drive the reaction to completion. The ATP concentration was propor-
tional to the decrease in NADH concentration, which was measured by
the decrease in absorbency at 340 nm.

**Fructose-1,6-diphosphate**

Liver fructose-1,6-diphosphate was also determined enzymati-
cally (110). Frozen liver powder was added to a tared tube contain-
ing 6N perchloric acid and weighed. Sufficient perchloric acid was
added so that there was a ratio of eight grams perchloric acid to
one gram liver. The homogenate was well mixed and centrifuged at
3000 xg for ten minutes. The extract was titrated with potassium
carbonate to pH 3.5 using methyl orange as an indicator. This was
allowed to stand ten minutes in the cold and then centrifuged to
remove the potassium perchlorate. The fructose-1,6-diphosphate was
assayed using the reaction sequence:

\[
\text{Fructose-1,6-diphosphate} \xleftarrow{\text{Aldolase}} \text{Dihydroxyacetone Phosphate} +
\text{Glyceraldehyde-3-Phosphate}
\]

\[
\text{Dihydroxyacetone Phosphate} \xleftarrow{\text{Triose Isomerase}} \text{Glyceraldehyde-3-Phosphate}
\]
Glyceraldehyde-3-Phosphate + NADH + H⁺ → Glycerol Phosphate

Dehydrogenase → NAD⁺ + Glycerol-1-Phosphate

The change in absorbency at 340 μm was determined and fructose-1,6-diphosphate concentration was calculated using the extinction coefficient of NADH at this wavelength.

Glycerol-1-phosphate

Glycerol-1-phosphate was determined on the same neutralized perchloric acid extract used for the determination of fructose-1,6-diphosphate (111). The supernate was incubated with NAD⁺, glycerol phosphate dehydrogenase and hydrazine.

\[
\text{NAD}^+ + \text{Glycerol-1-phosphate} \xrightarrow{\text{Glycerol Phosphate Dehydrogenase}} \text{NADH} + \text{H}^+ + \text{Glyceraldehyde-1-Phosphate}
\]

The change in absorbence at 340 μm was determined and the concentration of glycerol-1-phosphate was calculated using the extinction coefficient of NADH at this wavelength.

Glycogen

For the determination of glycogen, frozen liver powder was added to a tared tube containing 30% KOH. The tube was weighed, placed in a boiling water bath for fifteen minutes and then allowed to cool to room temperature. The glycogen was precipitated with absolute ethanol, washed three times with ethanol and then hydrolyzed by heating with 2N sulfuric acid for two hours. This effectively hydrolyzed all of the glycogen to glucose. The hydrolysate was
neutralized with 2N sodium hydroxide and diluted to 10 ml. with distilled water. The glucose concentration was determined using the Auto-Analyzer. When the glycogen concentrations were small, the hydrolysate glucose was measured using a micro glucose method adapted for the AutoAnalyzer. This method enabled us to measure glycogen concentrations of 0.1 mg./gram liver.

**DBI**

Liver and perfusate DBI concentrations were determined by the method of Freedman et al. (112). The perfusate protein was precipitated with 50% TCA and the DBI was extracted with alkaline chloroform-methanol. The extract was acidified, the solvent phase was evaporated and the residue containing DBI was dissolved in distilled water. Treatment with alpha-naphthol-diacetyl reagent produced a red color which was measured at 565 μm. For the determination of liver DBI, the tissue was homogenized in 20% sodium chloride and the protein was precipitated with TCA. The mixture was extracted with alkaline chloroform-methanol and the extract was passed through a Zeolite column. DBI was eluted with 5% sodium chloride and treated with alpha-naphthol-diacetyl color reagent. The method was capable of detecting as little as 5 μg DBI.
CHAPTER IV

RESULTS

Effect of free fatty acids on glucose release by perfused livers

Livers from fed animals were perfused alternately with high and low free fatty acid albumin solutions. Figure 3 shows the results obtained in a typical experiment. In this case glucose concentrations were 100 mg.% at the beginning of each hour. Increases in glucose concentration represent glucose output by the liver and decreases represent glucose uptake. It can be seen that free fatty acids stimulated glucose output by the liver. Figure 4 summarizes the results of a similar experiment in which the high free fatty acid concentration was 1600 μEq./l. This fatty acid concentration is one which might be expected to arise in a physiological situation. In the next experiment shown in Figure 5, the initial glucose concentration was set at 200 mg.% Glucose uptake can be observed during perfusion with low free fatty acid solutions; the uptake was virtually abolished during high free fatty acid perfusion. Figure 6 shows the results obtained when the initial glucose concentration was 150 mg.% It can be seen that although there was considerable variation from one liver to another, the alternation in glucose output caused by varying the free fatty concentration was always evident. In each of these experiments there
FIGURE 3

EFFECT OF FFA ON RELEASE OF GLUCOSE FROM THE LIVER OF A FED RAT
FIGURE 4

EFFECT OF FFA ON GLUCOSE RELEASE BY THE LIVER OF A FED RAT

Glucose (mg.%)

175
150
125
100

3000

1000

FFA (uEq./L)

1
2
3
4
5
6
7

Hours of Perfusion
FIGURE 5

EFFECT OF FFA ON GLUCOSE UPTAKE BY THE LIVER OF A FED RAT

Glucose (mg.%) vs. Hours of Perfusion

FFA (uEq./l) vs. Hours of Perfusion
FIGURE 6

THE EFFECT FFA ON GLUCOSE RELEASE BY THE LIVER OF A FED RAT

Hours of Perfusion

Glucose (mg.%)  
400
300
200
100

FFA (uEq/l)
2000
1000

was excessive glucose release during the first hour of perfusion irrespective of fatty acid level. This was probably due to a variety of factors including ether anesthesia and surgical manipulation.

**Liver phosphorylase activity**

Since the alternation in glucose output was obtained with livers from fed rats, it was postulated that perfusion with high concentrations of free fatty acids activated phosphorylase. A liver was perfused alternately with high and low free fatty acid solutions. At the end of each hour of perfusion a small biopsy (200 mg.) was taken and assayed in duplicate for phosphorylase activity. Epinephrine was added at the end of the experiment to test the liver's response to a known glycogenolytic compound. The results are shown in Figure 7. It can be seen that phosphorylase values tended to be higher during the periods of high fatty acid perfusion and that epinephrine caused a marked increase in phosphorylase activity. However, these biopsies were taken at the end of each hour when, even during high free fatty acid periods, glucose output by the liver had begun to level off and the fatty acid concentration had fallen considerably.

In another experiment the liver of a fed animal was perfused with low free fatty acid albumin. A tissue sample was removed when the glucose concentration in the perfusate had begun to fall slightly. Aqueous sodium octanoate was then added, bringing the free fatty acid concentration up to approximately 3000 µEq./l. A
FIGURE 7

EFFECT FFA ON PHOSPHORLASE ACTIVITY IN THE LIVER FROM A FED RAT

Hours of Perfusion

<table>
<thead>
<tr>
<th>Hours</th>
<th>Phosphorylase (uM Pi/g./min.)</th>
<th>Glucose (mg.%)</th>
<th>FFA (uEq./l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.10</td>
<td>150</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>2.51</td>
<td>150</td>
<td>2000</td>
</tr>
<tr>
<td>3</td>
<td>1.75</td>
<td>150</td>
<td>3000</td>
</tr>
<tr>
<td>4</td>
<td>2.45</td>
<td>150</td>
<td>2000</td>
</tr>
<tr>
<td>5</td>
<td>2.60</td>
<td>150</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>9.26</td>
<td>150</td>
<td>1000</td>
</tr>
</tbody>
</table>

Epinephrine
second biopsy was taken when the glucose concentration began to rise. Phosphorylase activity was determined in duplicate on both tissue samples. The results are shown in Figure 8. Phosphorylase activity increased by 62% with the addition of sodium octanoate. Figure 9 shows the results of a similar experiment. In this case sodium oleate was added to the perfusate following the first biopsy. Biopsy two was removed thirty minutes after the addition of oleate; at this time the glucose concentration in the perfusate was rising. A third biopsy was taken one hour later. At this time the fatty acid concentration had fallen to approximately 1000 µEq./l., glucose output had ceased and phosphorylase activity had fallen from 3.00 to 1.54 units.

Ketogenesis and lactate pyruvate ratios

Perfusion with high concentrations of free fatty acids caused a much greater production of ketone bodies than during periods of low free fatty acid perfusion (Table 1). Fatty acids also influenced lactate production and lactate/pyruvate ratios in the livers of fed rats. During periods of high fatty acid perfusion, lactate/pyruvate ratios were elevated whereas the lactate concentration itself was decreased. Table 2 presents the results from a typical experiment.

The livers from fasted rats, however, did not show changes in lactate production or lactate/pyruvate ratios. During both high and low free fatty acid periods the lactic acid production and
FIGURE 8

EFFECT OF FFA ON PHOSPHORYLASE ACTIVITY OF THE LIVER
FROM A FED RAT

Phosphorylase (uM Pi/g/min.)

200

150

100

50

Glucose (mg.%) 1.78 2.88

FFA (uEq./l.)

3000

2000

1000

100

50

1

2

3

Hours of Perfusion
FIGURE 9

EFFECT OF FFA ON PHOSPHORYLASE ACTIVITY OF THE LIVER FROM A FED RAT

Phosphorylase (uM Pi/g./min.)

Glucose (mg. %)

FFA (uEq./l.)

Sodium Oleate

Hours of Perfusion

100

150

3000

2000

1000

1.10

3.00

1.54

1

2

3
### TABLE 1

**EFFECT OF FFA ON KETONE BODY PRODUCTION**

*(FED RAT)*

<table>
<thead>
<tr>
<th>FFA</th>
<th>Hours</th>
<th>Total Ketones (μg/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low *</td>
<td>0.5</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>32.5</td>
</tr>
<tr>
<td>High **</td>
<td>1.5</td>
<td>53.4</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>97.6</td>
</tr>
<tr>
<td>Low</td>
<td>2.5</td>
<td>48.2</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>48.2</td>
</tr>
<tr>
<td>High</td>
<td>3.5</td>
<td>63.8</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>97.6</td>
</tr>
<tr>
<td>Low</td>
<td>4.5</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>23.4</td>
</tr>
</tbody>
</table>

* Initial low FFA perfusate contained 440 μEq./l. FFA
** Initial high FFA perfusate contained 2820 μEq./l. FFA
TABLE 2
EFFECT OF FFA ON LACTATE PRODUCTION AND LACTATE/PYRUVATE RATIOS
(FED RAT)

<table>
<thead>
<tr>
<th>FFA</th>
<th>Hours</th>
<th>Lactic Acid (mg.%)</th>
<th>Lactic Pyruvic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.5</td>
<td>10.6</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>20.0</td>
<td>9.7</td>
</tr>
<tr>
<td>High</td>
<td>1.5</td>
<td>5.4</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>5.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Low</td>
<td>2.5</td>
<td>7.0</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>11.5</td>
<td>5.4</td>
</tr>
<tr>
<td>High</td>
<td>3.5</td>
<td>5.9</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.0</td>
<td>17.6</td>
</tr>
<tr>
<td>Low</td>
<td>4.5</td>
<td>7.2</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>11.3</td>
<td>5.0</td>
</tr>
</tbody>
</table>
lactate/pyruvate ratios were similar to those seen when livers from fed animals were perfused with high levels of free fatty acids (Table 3). In addition, when the perfusate consisted of buffer containing no free fatty acids, livers from fasted animals produced much greater quantities of ketone bodies than did the livers from fed animals. These results are summarized in Table 4. Livers from fasted animals contained very little glycogen and did not release glucose when perfused with high concentrations of free fatty acids.

Gluconeogenesis

The effects of fatty acids on gluconeogenesis were studied in the next series of experiments. Livers from rats fasted 20 to 24 hours were perfused alternately with high and low free fatty acid media which contained constant amounts of glucose precursors. Figure 10 presents the results obtained when the perfusate contained 60 mg.% lactate. It is evident that free fatty acids enhanced the conversion of lactate to glucose. Similar results were obtained when pyruvate was employed as the gluconeogenic substrate (Figure 11). Fatty acids did not affect the rate of gluconeogenesis from glycerol (Figure 12). There appeared to be a slight fatty acid enhancement of gluconeogenesis from oxaloacetate (Figure 13). However, some of the oxaloacetate was apparently converted to lactate since the level of this metabolite was elevated. This lactate disappeared more rapidly in the presence of high levels of free fatty acids (Table 5).
**TABLE 3**

**EFFECT OF FFA ON LACTATE PRODUCTION AND LACTATE/PYRUVATE RATIOS**

*(FASTED RAT)*

<table>
<thead>
<tr>
<th>FFA</th>
<th>Hours</th>
<th>Lactate Mg.%</th>
<th>Lactate Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.5</td>
<td>6.31</td>
<td>42.1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6.04</td>
<td>28.8</td>
</tr>
<tr>
<td>High</td>
<td>1.5</td>
<td>8.74</td>
<td>48.6</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>6.76</td>
<td>37.6</td>
</tr>
<tr>
<td>Low</td>
<td>2.5</td>
<td>5.32</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>5.41</td>
<td>30.1</td>
</tr>
<tr>
<td>High</td>
<td>3.5</td>
<td>5.41</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>5.32</td>
<td>22.2</td>
</tr>
<tr>
<td>Low</td>
<td>4.5</td>
<td>3.87</td>
<td>25.8</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>3.60</td>
<td>20.0</td>
</tr>
</tbody>
</table>

* Initial low 440 µEq./l. FFA
** Initial high 2820 µEq./l. FFA
TABLE 4

KETOGENESIS BY LIVERS FROM FED AND FASTED RATS (NO ADDED FFA)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Total Ketones (µg./ml.)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hours</td>
<td>1 hour</td>
<td>2 hours</td>
</tr>
<tr>
<td>Fed</td>
<td>7.9 ± 3.8 (6)*</td>
<td>18.2 ± 9.1 (6)</td>
<td></td>
</tr>
<tr>
<td>Fasted 24 hours</td>
<td>45.0 ± 28.6 (3)</td>
<td>108.4 ± 28.6 (3)</td>
<td></td>
</tr>
<tr>
<td>Fasted 48 hours</td>
<td>56.3 ± 27.4 (7)</td>
<td>108.2 ± 40.5 (7)</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers in parentheses denote number of experiments
FIGURE 10

EFFECT OF FFA ON GLUCONEOGENESIS FROM LACTATE (60 mg.%) BY THE LIVER FROM A FASTED RAT
FIGURE 11

EFFECT OF FFA ON GLUCONEOGENESIS FROM PYRUVATE (60 mg.%) BY THE LIVER FROM A FASTED RAT

Glucose (mg. %)

Hours of Perfusion

Hours of Perfusion

FFA (uEq./l.)

160
140
120
100
3000
2000
2000
1000
FIGURE 12

EFFECT OF FFA ON GLUCONEOGENESIS FROM GLYCEROL (35 mg. %) 
BY THE LIVER FROM A FASTED RAT

Glucose (mg. %)

140
120
100
80

FFA (μEq./l.)

3000
2000
1000

Hours of Perfusion

1 2 3 4 5
EFFECT OF FFA ON GLUCONEOGENESIS FROM OXALOACETATE (100 mg.%)
BY THE LIVER FROM A FASTED RAT

FIGURE 13

Glucose (mg. %)

FFA (uEq./l)

1000

140

120

100

2000

1000

Hours of Perfusion

1 2 3 4 5
TABLE 5

EFFECT OF FFA ON THE UTILIZATION OF LACTATE AND PYRUVATE PRODUCED DURING PERFUSION WITH OXALOACETATE (100 mg.%)

<table>
<thead>
<tr>
<th>FFA</th>
<th>Hours</th>
<th>Lactate* mg.%</th>
<th>Pyruvate mg.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.5</td>
<td>19.10</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10.36</td>
<td>2.32</td>
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<tr>
<td>High</td>
<td>1.5</td>
<td>18.29</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.61</td>
<td>0.12</td>
</tr>
<tr>
<td>Low</td>
<td>2.5</td>
<td>22.98</td>
<td>7.50</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>21.80</td>
<td>4.26</td>
</tr>
<tr>
<td>High</td>
<td>3.5</td>
<td>23.16</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.05</td>
<td>0.18</td>
</tr>
<tr>
<td>Low</td>
<td>4.5</td>
<td>21.89</td>
<td>6.72</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>27.03</td>
<td>3.88</td>
</tr>
</tbody>
</table>

* The initial lactate concentration was 2.6 mg.% and the initial pyruvate concentration was 7.0 mg.%.
In order to investigate glucose output in the presence and absence of substrate, high (60 mg.%) and low (0.5 mg.%) concentrations of lactate were alternately perfused through the liver of a fasted animal. Both media contained high concentrations of free fatty acids. The effects on glucose output by the liver are shown in Figure 14. A similar experiment (Figure 15) was performed using high fat media containing 50 mg.% pyruvate alternated with high fat media containing no gluconeogenic substrate. It can be seen that, even in the presence of high concentrations of free fatty acids, livers from fasted animals put out very little glucose in the absence of added substrate.

The effect of free fatty acids on gluconeogenesis was also studied by measuring the incorporation of label from $^{14}$C-lactate into glucose and glycogen. The results, shown in Figure 16 indicate that gluconeogenesis was greatly enhanced by free fatty acids in the livers of fed rats. Gluconeogenesis was also enhanced in the livers from fasted rats, however, the effect was not as great since the baseline level was very high. In the presence of high levels of free fatty acids, there was little difference in the rate of gluconeogenesis between livers from fed and fasted animals.

Fatty acids enhanced not only the conversion of lactate to glucose but the uptake of lactate by the liver as well. In the livers from fed rats, free fatty acids also increased the percentage of this lactate which was converted to glucose and glycogen (Table 6).
FIGURE 14

EFFECT OF LACTATE ON GLUCOSE OUTPUT BY THE LIVER FROM A FASTED RAT
(ALL HIGH FFA)

Glucose (mg.%) vs. Lactate (mg.%) over 5 hours of perfusion.
EFFECT OF PYRUVATE ON GLUCOSE OUTPUT BY THE LIVER FROM A FASTED RAT (ALL HIGH FFA)
FIGURE 16

EFFECT OF FFA ON INCORPORATION OF $^{14}$C-LACTATE INTO GLUCOSE
BY ISOLATED PERFUSED RAT LIVERS

Mg. $^{14}$C-Lactate
Converted to
Glucose/Hour

FFA (uEq./l.)

$\Delta$ — Fasted
$\circ$ — Fed
TABLE 6
EFFECT OF FFA ON GLUCONEOGENESIS FROM $^{14}$C-LACTATE

<table>
<thead>
<tr>
<th>Animal</th>
<th>FFA</th>
<th>14C-Lactate</th>
<th>Mg. Lactate Taken up by Liver</th>
<th>Percentage of Removed Lactate Converted to Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incorporated into 14C-Glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment</td>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Fed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>660</td>
<td>1.9</td>
<td>1.4</td>
<td>19.3</td>
</tr>
<tr>
<td></td>
<td>1360</td>
<td>7.1</td>
<td>6.9</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td>3450</td>
<td>11.1</td>
<td>15.0</td>
<td>31.9</td>
</tr>
<tr>
<td>Fasted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>13.1</td>
<td>9.7</td>
<td>38.7</td>
</tr>
<tr>
<td></td>
<td>3450</td>
<td>14.2</td>
<td>11.2</td>
<td>46.0</td>
</tr>
</tbody>
</table>
Effects of DBI

Numerous attempts were made to demonstrate an effect of DBI on gluconeogenesis in perfused rat livers. The only consistent finding was that perfusion with DBI concentrations of 50 mg.% or more caused an outpouring of glucose from glycogen containing livers. No inhibition of gluconeogenesis could be demonstrated with DBI concentrations as high as 50 mg.% (Figure 17). When doses of DBI up to 200 mg./kg. were administered orally to fasted rats it was discovered that, contrary to the results of Ungar et al. (81), DBI did not cause hypoglycemia. When DBI was given per os to fed rats a marked hyperglycemia resulted. Guinea pigs, on the other hand, became profoundly hypoglycemic when 50 mg./kg. DBI was given orally.

The effects of DBI were studied using isolated perfused guinea pig livers. These livers were perfused with Krebs-Ringer buffer rather than with the 5% albumin solution in order to eliminate the effects of free fatty acids. Figure 18 shows the results of a typical experiment in which a guinea pig liver was perfused with 50 mg.% DBI. It can be seen that the conversion of pyruvate to glucose was inhibited by DBI, and this inhibition was not relieved by perfusing with fresh medium containing no drugs. The inhibition by DBI of gluconeogenesis from glycerol is depicted in Figure 19. In this instance also, perfusion with fresh media did not relieve the inhibition of gluconeogenesis. In fact, the inhibition was greatest two hours after the perfusion with DBI ceased.
FIGURE 17
EFFECT OF DBI ON GLUCONEOGENESIS FROM LACTATE IN THE PERFUSED LIVER FROM A FASTED RAT
FIGURE 18

EFFECT DBI ON GLUCONEOGENESIS FROM PYRUVATE IN THE ISOLATED LIVER FROM A FASTED GUINEA PIG

Glucose (mg.%)  
Lactate ----  
Pyruvate ---  

Hours of Perfusion
FIGURE 19

EFFECT OF DBI ON GLUCONEOGENESIS FROM GLYCEROL BY AN ISOLATED LIVER FROM A FASTED GUINEA PIG

![Graph showing the effect of DBI on gluconeogenesis from glycerol by an isolated liver from a fasted guinea pig. The graph plots glucose and glycerol levels in mg.% over 4 hours of perfusion. The graph indicates a decrease in glycerol levels and an increase in glucose levels after exposure to 10 mg.% DBI.](image)
A dose response curve to DBI is presented in Figure 20. The per cent inhibition of gluconeogenesis from glycerol is plotted against the DBI concentration. Per cent inhibition was obtained by comparing the amount of glucose released during the hour prior to drug administration with the amount released during the first hour following the hour long perfusion with DBI. It is evident that inhibition of gluconeogenesis was a function of the amount of DBI used.

In an attempt to elucidate the mechanism of action of DBI, the concentrations of a number of metabolic intermediates were determined. It can be seen from Figure 21 that during perfusion of guinea pig livers with glycerol, DBI caused both fructose-1,6-diphosphate and glycerol-1-phosphate to increase when relatively low levels of DBI were used (5 mg.%). At higher levels of DBI the concentrations of these metabolites decreased. Perfusion with oxaloacetate and pyruvate also resulted in elevated levels of fructose-1,6-diphosphate in the presence of DBI. However, the results were extremely variable.

Since ATP is essential for gluconeogenesis, the effect of DBI on ATP concentration in perfused rat and guinea pig livers was determined. It can be seen in Table 7 that guinea pig liver ATP levels fell markedly in the presence of DBI whereas there was no effect on rat liver ATP. In order to determine whether this in vitro effect also occurred in vivo, varying doses of DBI were
FIGURE 20

INHIBITION OF GLUCONEOGENESIS FROM GLYCEROL BY DBI IN LIVERS FROM FASTED GUINEA PIGS

% Inhibition

Mg. % DBI

-20

0

20

40

60

80

100
FIGURE 21

EFFECT OF DBI ON GLYCEROL-1-PHOSPHATE
AND FRUCTOSE-1,6-DIPHOSPHATE IN LIVERS
FROM FASTED GUINEA PIGS PERFUSED WITH GLYCEROL
TABLE 7

EFFECT OF DBI ON ATP CONCENTRATIONS
IN PERFUSED LIVERS

<table>
<thead>
<tr>
<th>Animal</th>
<th>DBI mg.%</th>
<th>ATP µM/g. (wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>2.50 (4) ± 0.15</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>2.00 (1)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1.87 (3) ± 0.10</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.19 (1)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.61 (1)</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>2.43 (1)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>2.45 (1)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>2.34 (1)</td>
</tr>
</tbody>
</table>

* Number of samples
administered subcutaneously as an aqueous solution to fasted guinea pigs and rats. Exactly two hours later the animals were anesthetized with ether and a sample of heart blood was drawn for a glucose determination. The livers of these animals were rinsed out with oxygenated Krebs-buffer, quickly frozen between liquid nitrogen cooled clamps and assayed in duplicate for ATP concentration. The results are presented in Figure 22. It is apparent that low levels of ATP are associated with hypoglycemia. In addition, both blood glucose and liver ATP fell dramatically in guinea pigs but not in rats.

The difference in response to DBI between guinea pigs and rats could not be explained on the basis of a difference in the uptake of this drug. Table 8 presents the results of an experiment in which guinea pig liver and a rat liver were perfused with 50 mg.% DBI. It can be seen that the uptake per gram of tissue of this drug by rat and guinea pig livers was practically identical. At the end of the experiment approximately twice as much DBI was recovered from the rat liver per gram of tissue compared to that recovered from the guinea pig liver.
FIGURE 22

EFFECT OF DBI ON BLOOD GLUCOSE AND LIVER ATP IN VIVO

Blood Glucose (mg.%) vs. DBI (mg./kg.)

- Rat
- Guinea Pig

ATP (μM/g.) vs. DBI (mg./kg.)
TABLE 8

UPTAKE OF DBI BY RAT AND GUINEA PIG LIVERS

<table>
<thead>
<tr>
<th>Animal</th>
<th>Time (Hours)</th>
<th>mg.% DBI</th>
<th>mg. DBI removed/gram liver</th>
<th>mg. DBI recovered/gram liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>0</td>
<td>47.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>40.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>36.0</td>
<td>1.6</td>
<td>0.52</td>
</tr>
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<td>3</td>
<td>36.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>36.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea pig</td>
<td>0</td>
<td>47.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>31.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.5</td>
<td>1.7</td>
<td>0.26</td>
</tr>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>27.5</td>
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</table>
CHAPTER V

DISCUSSION

Glucose and fatty acid metabolism appear to be inter-related in such a way that blood glucose concentrations regulate the release of free fatty acids from adipose tissue and free fatty acid concentrations in turn, exert a regulatory effect on glucose release from the liver. Thus, the glucose-fatty acid cycle of Randle et al. (1) can be expanded to include the liver as well as muscle and adipose tissue. Figure 23 is a representation of this expanded glucose-fatty acid cycle. High levels of circulating glucose suppress the release of free fatty acids from adipose tissue by enhancing their reesterification. Therefore, when blood glucose levels increase, free fatty acids tend to decrease. Conversely, when blood glucose levels decrease, free fatty acid concentrations increase. The increased levels of free fatty acids not only suppress glucose uptake by muscle and adipose tissue as pointed out by Randle; they also stimulate the release of glucose from the liver. We have shown that this increased glucose release is brought about by enhancement of gluconeogenesis and by activation of phosphorylase.

High levels of free fatty acids caused glucose to be released from the livers of fed rats when the perfusate glucose
FIGURE 23

THE GLUCOSE-FATTY ACID CYCLE

Liver

GLUCONEOGENESIS

GLYCOGEN

Muscle

Adipose

TRIGLYCERIDE

Liver

GLUCONEOGENESIS

GLYCOGEN

Muscle

Adipose

TRIGLYCERIDE

Muscle

FFA

Muscle

GLUCOSE

Muscle

KCO
concentrations were 100 and 150 mg%. When these livers were perfused alternately with high and low free fatty acid media, glucose was released during the high free fatty acid periods whereas there was little or no release of glucose during periods of low free fatty acid perfusion (Figures 3, 4 and 6). With an initial glucose concentration of 200 mg.% glucose was taken up during periods of low free fatty acid perfusion; this uptake was abolished during periods of high free fatty acid perfusion (Figure 5). These effects could not be obtained using livers from fasted rats. We therefore postulated that perfusion of livers from fed rats with high concentrations of free fatty acids activated phosphorylase.

Figures 7, 8 and 9 show that the addition of free fatty acids to the perfusate caused an outpouring of glucose from the liver of a fed rat. This was accompanied by almost a doubling of phosphorylase activity. According to Figure 9, when the free fatty acid concentration of the perfusate had fallen to approximately 1000 µEq./l. and the outpouring of glucose had ceased, the phosphorylase activity had fallen almost to the original level. The effects of free fatty acids on glucose output by the fed liver can then be attributed to an activation of phosphorylase.

The effects of fatty acids on phosphorylase activity and glycogenolysis in ruminant livers have recently been investigated. Phillips et al. (112) demonstrated that the infusion of sodium butyrate into adrenalectomized lambs caused a prompt doubling of
liver phosphorylase activity accompanied by hyperglycemia and depletion of liver glycogen. This effect could not be duplicated by injecting sodium acetate or beta-hydroxybutyrate into lambs. These workers were unable to rule out the possibility that the effects of butyrate were indirect, being mediated through the action of some other compound such as glucagon, rather than being direct effects on phosphorylase since they were working with the whole animal.

The increase in phosphorylase activity observed in lamb liver in vivo was of the same order of magnitude as the increase we observed with rat liver in vitro. However, our results were not entirely comparable to those reported with ruminant livers since we could demonstrate no effect of butyrate on glucogenolysis in the rat liver. We obtained these effects with caprylate, oleate, and linoleate. Butyrate is a normal product of ruminant digestion whereas butyrate rarely is produced during digestion in the rat. It may be that butyrate oxidation is more rapid in sheep liver than in rat liver.

Ash et al. (113) investigated the effects of free fatty acid infusions on blood glucose levels in sheep. They too observed a hyperglycemic response with butyrate but not with acetate or beta-hydroxybutyrate. These investigators also obtained a hyperglycemic response following the injection of propionate and hexanoate. Labeling studies demonstrated that the increased glucose production was not the result of gluconeogenesis from short chain fatty acids.
Propionic acid can be converted to glucose but this conversion was not rapid enough to account for the increase in blood glucose. These authors were unable to show an effect of butyrate on glycogenolysis in sheep liver slices.

In 1948, Nath and his co-workers (114) reported that the incubation of rat liver slices with 2 and 5 mg. acetoacetate accelerated glycogenolysis 44% and 70% respectively. It may be that the hyperglycemia which we observed was mediated by acetoacetate. This would be consistent with the findings that fatty acids stimulated glycogenolysis whereas beta-hydroxybutyrate and acetate did not.

The activation of phosphorylase by free fatty acids provides a mechanism whereby liver glycogen is slowly broken down to provide blood glucose during short periods of fasting. This would be important for the regulation of blood glucose levels in animals which feed intermittently. However, the glycogen reserves of the animal are not adequate to provide sufficient blood glucose to the fasting animal. During fasting, blood glucose is derived primarily from non-carbohydrate substances which are converted to glucose by the liver and kidney. We have demonstrated that gluconeogenesis, as well as glycogenolysis, is controlled in part by free fatty acids.

Fatty acids markedly enhanced gluconeogenesis from lactate and pyruvate but had no effect on gluconeogenesis from glycerol in the isolated perfused rat liver. This fact suggested that free
fatty acids stimulate gluconeogenesis at some point prior to the triose phosphates. Since glycerol enters the gluconeogenic pathway at the level of the triose phosphates, gluconeogenesis from glycerol was not affected by free fatty acids (Figure 24). According to Krebs (2), the rate-limiting step between pyruvate and triose phosphate involves the conversion of pyruvate to phosphoenolpyruvate. This conversion proceeds in two steps. Pyruvate is first converted to oxaloacetate by pyruvic carboxylase, CO$_2$ and ATP.

$$\text{Pyruvate} + \text{CO}_2 + \text{ATP} \xrightarrow{\text{Pyruvic Carboxylase}} \text{Oxaloacetate} + \text{ADP}$$

Oxaloacetate is then converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase:

$$\text{Oxaloacetate} + \text{GTP} \xrightarrow{\text{PEP Carboxykinase}} \text{PEP} + \text{GDP} + \text{CO}_2$$

Since pyruvic carboxylase requires acetyl CoA as an allosteric activator, we postulate that fatty acids enhance this reaction by generating acetyl CoA. To test this hypothesis we compared the rates of gluconeogenesis from oxaloacetate obtained by perfusing rat liver with high and low free fatty acid media. There was practically no fatty acid enhancement of gluconeogenesis from oxaloacetate (Figure 13). It therefore seems likely that fatty acids stimulated the pyruvic carboxylase reaction, probably by generating acetyl CoA.

The $K_m$ of pyruvic carboxylase for acetyl CoA was shown to be approximately 0.02 mM (2). Wieland reported that the acetyl CoA concentration in normal livers was roughly 0.02 mM (57). This was increased to 0.047 and 0.058 mM in livers from diabetic and fat fed
FIGURE 24

GLUCONEOGENIC AND GLYCOLOYTIC PATHWAYS

Lactate

NAD

NADH₂

Pyruvate

NADH₂

ATP

ADP

GTP

Oxaloacetate

GDP

CO₂

Phosphopyruvate

+H₂O

H₂O

2-phosphoglycerate

3-phosphoglycerate

ATP

ADP

1,3-diphosphoglycerate

NADH₂

NAD

Glyceraldehydephosphate

Dihydroxyacetone phosphate

Fructose-1,6-diphosphate

Pi

Fructose-6-phosphate

Glucose-6-phosphate

Pi

Glucose

ATP

ADP
rats respectively. Thus the acetyl CoA concentrations observed during the increased fatty acid oxidation associated with diabetes and fat feeding are within the range necessary for the activation of pyruvic carboxylase.

There was a slight fatty acid enhancement of gluconeogenesis from oxaloacetate, but this was only a fraction of that seen with lactate and pyruvate. This slight enhancement can be accounted for in several ways. The data of Table 5 clearly show that some of the added oxaloacetate was converted to lactate. The subsequent gluconeogenesis from this substrate was enhanced by free fatty acids.

The metabolism of fatty acids may have other stimulatory effects on gluconeogenesis. Weber (72) has shown that NADH inhibits pyruvic kinase, the enzyme which catalyzes the conversion of PEP + ADP to pyruvate and ATP. It is conceivable that free fatty acids enhance gluconeogenesis by generating NADH which could inhibit the breakdown of PEP.

Free fatty acids can exert a sparing effect on lactate and pyruvate oxidation. According to the data of Table 6, fatty acids not only enhanced the uptake of lactate by the liver but also increased the percentage of this removed lactate which was converted to glucose. Wieland et al. (57) have shown that fatty acyl CoA inhibits the citrate synthetase reaction. Therefore, fatty acids can inhibit the consumption of oxaloacetate by the Krebs cycle and
divert this oxaloacetate to gluconeogenesis. In the presence of excess oxaloacetate this would not affect the rate of gluconeogenesis but at physiologic concentrations of oxaloacetate it may be important.

Free fatty acids can enhance gluconeogenesis from pyruvate by activating pyruvic carboxylase, by exerting a sparing effect on the oxaloacetate generated in this reaction, and possibly, by inhibiting the breakdown of PEP. This could effectively drive the conversion of pyruvate to PEP and prevent the reverse reaction (Figure 25). The oxidation of fatty acids can also provide the ATP and NADH necessary to gluconeogenesis.

Our results indicated that the effect of free fatty acids on gluconeogenesis was greatest in livers from fed rats since fasting rats had a high baseline level of gluconeogenesis. The livers from fasted rats produced approximately six times the quantity of ketone bodies in the absence of added fatty acids compared to livers from fed rats (Table 4). In addition to this, the perfusate lactate/pyruvate ratios obtained when fasted livers were perfused with buffer containing no fatty acids were extremely high (Table 3). Such high ratios were also obtained when the livers from fed animals were perfused with high fat media (Table 2).

These results suggested that the livers from fasted animals rapidly oxidized endogenous lipids. Mosinger and Varinkova (116) have presented evidence that liver lipase is increased during fasting. They demonstrated that free fatty acids accumulated in
THE EFFECT OF FFA ON THE CONVERSION OF PYRUVATE TO PHOSPHOENOLPYRUVATE

\[
\text{FFA} \rightarrow \text{FFA-CoA} \rightarrow \text{Acetyl CoA} + \text{NADH}
\]

\[
\text{ADP} \rightarrow \text{ATP}
\]

\[
\text{Lactate} \rightarrow \text{Pyruvate} \rightarrow \text{Oxaloacetate} \rightarrow \text{CO}_2 \rightarrow \text{CO}_2 \rightarrow \text{GTP} \rightarrow \text{GDP} \rightarrow \text{GLUCOSE}
\]

\[
\text{Amino acids} \rightarrow \text{Pyruvate} \rightarrow \text{Pyruvic Carboxylase} \rightarrow \text{Oxaloacetate} \rightarrow \text{PEP carboxykinase} \rightarrow \text{Phosphoenolpyruvate} \rightarrow \text{GLUCOSE}
\]

\[
\text{Pyruvic Kinase} \rightarrow \text{ATP} \rightarrow \text{ADP}
\]
rat liver slices which were incubated with cyanide. Cyanide was used to prevent reesterification and oxidation of the free fatty acids. The accumulation of fatty acids was more than twice as great in slices from fasted rats than in slices from rats fed ad libitum. The difference could not be accounted for on the basis of differences in liver fat (4.3% for fed and 4.7% for fasted) nor were the differences eliminated by calculating free fatty acids liberated per liver rather than per gram of tissue.

In addition to this intracellular lipolytic activity liver slices were able to hydrolyze lipid emulsions. The ability to hydrolyze lipid emulsions was decreased during fasting. Mosingler and Varinkova concluded that there are at least two liver lipases, one of which is an intracellular enzyme. An increase in this intracellular lipase during fasting would help to explain the increased ketogenesis and increased lactate/pyruvate ratios which we observed. We would like to suggest further than the high baseline level of gluconeogenesis which we observed in fasted livers is due in part to the increased oxidation of endogenous lipids. The importance of this increased intracellular lipase in fasted livers is difficult to assess since plasma free fatty acids are also increased during fasting.

Free fatty acids appear to exert a fine control over blood glucose concentration. Gradual decreases in blood glucose during fasting would permit the release of more free fatty acids from
adipose tissue. We have shown that these fatty acids can stimulate glycogenolysis and gluconeogenesis. Both of these processes would cause the gradual release of glucose into the circulation. The effect of free fatty acids on gluconeogenesis may also be of importance during strenuous exercise. During exercise muscle tissue releases great quantities of lactic acid which are derived from the anaerobic metabolism of glucose. There is also an increased liberation of free fatty acids from adipose tissue. These fatty acids would enhance the uptake of lactic acid by the liver and its subsequent conversion to glucose.

During prolonged periods of fasting several other adaptations take place to insure adequate blood glucose levels. The synthesis of the key gluconeogenic enzymes is increased or preferentially maintained and tissue protein is mobilized to provide the liver with gluconeogenic precursors. Krebs (2) termed these processes the coarse control of gluconeogenesis and contrasted them with the fine control exerted by various feedback mechanisms such as those which we described.

Weber has emphasized the role of glucocorticoids in maintaining blood glucose levels during fasting (71, 72). He postulated that the key gluconeogenic enzymes comprise a functional genome which is induced or repressed as a unit. He suggested that the glucocorticoid hormones were inducers of gluconeogenic enzyme synthesis and that insulin suppressed this synthesis. He demonstrated that glucose-6-phosphatase and fructose-1,6-diphosphatase
were increased three days following daily injections of cortisol. This increase was abolished by insulin and by actinomycin D, which is an inhibitor of protein synthesis. He also showed that glucose-6-phosphatase activity increased during six days of starvation and that fructose-1,6-diphosphatase activity decreased only slightly when compared to cellular nitrogen or to the activities of enzymes not involved in gluconeogenesis (71). These effects were not observed in hypophysectomized animals. Weber has also demonstrated increased incorporation of \(^{14} \text{C}\)-orotate into RNA and increases in total RNA following the injection of triamcinolone, a synthetic steroid. This increase in RNA synthesis tends to support the hypothesis that glucocorticoids act on hepatic enzyme forming systems.

However, Kvam and Park (117) reported that the administration of sufficient ethionine to block increases in glucose-6-phosphatase and fructose-1,6-diphosphatase did not block all of the gluconeogenic effects of hydrocortisone. Shrago et al. (118) reported that fasting induced the formation of PEP carboxykinase in both normal and adrenalectomized rats. Ray, Foster and Lardy (73) presented evidence that the primary effect of glucocorticoids is exerted on existing enzyme systems to direct metabolites toward carbohydrate synthesis. They suggested that the induction of key gluconeogenic enzymes is a secondary effect which is necessary for the maximum expression of the hormone's activity.

That the adrenals are necessary for the maintenance of blood glucose during prolonged periods of fasting has been recognized for
many years. However, whether changes in the levels of these hormones control gluconeogenesis is another question. There is some doubt that the glucocorticoids are increased during fasting (119). The absence of insulin secretion may simply allow these hormones to express their full activity.

The fact that fructose-1,6-diphosphatase is "preferentially maintained" during fasting rather than actually being increased would cause one to doubt that changes in the levels of the key gluconeogenic enzymes are responsible for the increased gluconeogenesis seen during fasting. It seems more likely that the glucocorticoids maintain the levels of the key gluconeogenic enzymes and mobilize tissue protein to provide a substrate for gluconeogenesis. These hormones also increase the activities of the enzymes involved in the breakdown of amino acids (73). High levels of free fatty acids are, however, necessary for maximum glucose production.

We also investigated the effects of DBI on glucose production. Since its synthesis by Ungar and his group (81) in 1957, DBI has been used with some success as an oral hypoglycemic agent. However, its mechanism of action has not yet been clearly elucidated. Several reports have indicated that DBI diminishes hepatic glucose release. We therefore investigated the effects of DBI on glucose release from isolated perfused rat livers. All attempts to demonstrate decreased glucose output in the presence of DBI were unsuccessful (Figure 17). Large concentrations of DBI (50 mg.%)
caused increased glucose output from livers of fed animals. This was probably due to glycogenolysis since the increased glucose output occurred in the absence of added gluconeogenic substrate. We subsequently discovered that, contrary to the results of Ungar et al. (81), DBI concentrations up to 200 mg./kg. did not cause hypoglycemia in normal rats. In fact, DBI caused hyperglycemia in rats which had been fed ad libitum.

Guinea pigs, on the other hand, became hypoglycemic following the administration of DBI. We therefore investigated the effects of DBI on glucose release from isolated perfused guinea pig livers. Gluconeogenesis from lactate, pyruvate, glycerol, and oxaloacetate was inhibited by DBI. This inhibition was a function of the concentration of DBI in the perfusate and was not relieved by perfusing the livers with fresh media containing no DBI. In fact, the inhibition increased steadily each hour following that hour during which DBI was administered (Figures 18, 19).

DBI has been shown to be a respiratory inhibitor, blocking the reaction between ADP and the high energy containing respiratory chain, thus blocking the generation of ATP (93). Since ATP is necessary to gluconeogenesis, we investigated the effects of DBI on liver ATP both in vivo and in vitro. According to the data of Table 8, ATP concentrations in isolated perfused guinea pig livers were decreased by DBI whereas DBI had no effect on the concentration of ATP in isolated perfused rat livers. When DBI was administered
subcutaneously to fasted guinea pigs, there was a decrease in liver ATP two hours later which was accompanied by hypoglycemia. No such effects were obtained when DBI was administered to fasted rats (Figure 22).

It seemed likely that the hypoglycemic action of DBI was related somehow to its ability to decrease liver ATP. Fructose-1,6-diphosphatase, one of the key gluconeogenic enzymes, is extremely sensitive to AMP concentrations which are, in turn, closely related to ATP concentrations. Krebs (2) has emphasized that tissue concentrations of AMP are subject to much greater percentage changes than are the concentrations of ATP or ADP. ATP, ADP, and AMP are maintained in an equilibrium state by adenylate kinase:

$$\frac{(AMP)(ATP)}{(ADP)^2} = 0.44$$

Using Krebs' example (2), if we assume that the sum of the concentrations of the adenine nucleotides is 5 mM and that the concentration of ATP is 3.9 mM, then a 43% decrease in ATP concentration would be accompanied by a 100% increase in ADP concentration and a 720% increase in AMP. Slight decreases in ATP concentration cause marked increases in AMP concentration. Therefore the inhibition of ATP generation caused by DBI would be expected to elevate the concentration of AMP, and since AMP is a potent inhibitor of fructose-1,6-diphosphatase, it would indirectly inhibit the activity of this enzyme. Inhibition of fructose-1,6-diphosphatase by DBI
is consistent with the fact that DBI inhibited gluconeogenesis from lactate, pyruvate, oxaloacetate and glycerol, but did not interfere with glycogenolysis. The key gluconeogenic reactions which are common to the conversion of these four substrates to glucose are the fructose-1,6-diphosphatase and glucose-6-phosphatase reactions. However, glucose-6-phosphatase is involved in the conversion of glycogen to glucose and this conversion did not appear to be inhibited. We thus postulated that DBI inhibited fructose-1,6-diphosphatase by interfering with ATP production therefore causing the accumulation of AMP. If this reaction were inhibited one would expect the accumulation of fructose-1,6-diphosphate in the liver.

When lactate, pyruvate, and oxaloacetate were used as gluconeogenic substrates the average fructose-1,6-diphosphate concentration in the liver was approximately $13 \mu M/\text{gram of tissue}$. The addition of ten mg. DBI caused the average fructose-1,6-diphosphate concentration to increase to $18 \mu M/\text{gram of liver}$. However, the results were extremely variable due perhaps to the fact that fructose-1,6-diphosphate is in equilibrium with glyceraldehyde phosphate and dihydroxyacetone phosphate. The glyceraldehyde phosphate, in turn, can be readily converted to glycerol-1-phosphate. The extent of this conversion is a function of the $\text{NADH}_2/\text{NAD}$ ratio in the liver cell.

When glycerol was used as the glucose precursor, low levels of DBI (5 mg.%) caused increases in both fructose-1,6-diphosphate
and glycerol-1-phosphate. At higher DBI concentrations (10 mg/%) the levels of these metabolites fell. It may be that at higher concentrations of DBI there was insufficient ATP to drive the glycerol kinase reaction (Figure 21).

Our results indicate that the hypoglycemia produced in normal guinea pigs by DBI is the result of decreased gluconeogenesis. DBI inhibits gluconeogenesis by interfering with the production of ATP. Slight decreases in ATP concentrations are accompanied by increases in AMP sufficient to inhibit fructose-1,6-diphosphatase. When higher concentrations of DBI are used there may also be insufficient ATP for the various energy requiring reactions of gluconeogenesis. DBI does not produce hypoglycemia in normal rats nor does it lower liver ATP. However, much of the in vitro work on DBI has been performed with rat liver tissue.

DBI inhibits oxygen consumption in rat liver homogenates and in rat liver mitochondria. Rat and guinea pig livers remove DBI from the perfusate at comparable rates and similar amounts of DBI can be recovered from rat and guinea pig livers perfused with DBI. It is thus difficult to explain the lack of response to DBI shown by rats in vivo and by perfused rat livers in vitro.

Patrick (120) demonstrated slight decreases in rat liver ATP when he incubated liver slices with DBI, but his results were not statistically significant. However, rat kidney slices were responsive to DBI, exhibiting both decreases in ATP concentration
and in gluconeogenic ability. He could demonstrate no inhibition of glucose-6-phosphatase in kidney homogenates treated with DBI and concluded that DBI inhibited gluconeogenesis, at least in rat kidney, by lowering ATP levels. Further work is necessary to discover why guinea pigs become hypoglycemic when treated with DBI whereas rats do not.

The marked species differences in susceptibility to the hypoglycemic action of DBI make it difficult to relate findings obtained in guinea pigs and rats to the effects observed in humans following the administration of DBI. Normal humans, like rats, do not become hypoglycemic after the ingestion of DBI. However, DBI is effective in lowering blood glucose concentrations in diabetics.

There is evidence to suggest that DBI inhibits gluconeogenesis in diabetic humans. Moorehouse (121) and his co-workers observed that DBI caused large increases in fasting levels of lactate and pyruvate in human diabetics. DBI also inhibited the removal of a ten gram test dose of pyruvate from the circulation. Although this effect was much greater in diabetic patients, it was also evident in non-diabetics. These authors stated that the hypoglycemic effect of DBI was quantitatively related to the inhibition of pyruvate disposal. These data are consistent with the hypothesis that DBI inhibits gluconeogenesis. In our experiments with isolated perfused guinea pig livers, DBI inhibited the uptake of lactate and pyruvate by the liver as well as its conversion to glucose.
Searle (122) and his group recently investigated the effects of DBI on $^{14}$C-glucose turnover in non-diabetic patients. Although blood glucose levels remained fairly constant, $^{14}$C-glucose disappeared from the circulation more rapidly following the administration of DBI. These data were interpreted as evidence for the concept that DBI enhances peripheral utilization of glucose. However, these data could also be explained by the concept that DBI inhibits gluconeogenesis. Normally there would be much recycling of the $^{14}$C-label in glucose because glucose is readily metabolized by erythrocytes and muscle tissue. An important product of this metabolism, lactic acid, is reconverted to glucose by the liver. If DBI inhibited gluconeogenesis, the reconversion of labeled lactate to glucose would be inhibited. Hence the labeled glucose would disappear more rapidly from the circulation of non-diabetic patients receiving DBI.

It would be very interesting to repeat the pyruvate tolerance test of Moorehouse et al. (122) using $^{14}$C-labeled pyruvate. The effects of DBI on gluconeogenesis in normal and diabetic patients could be measured by determining the amount of incorporation of label into blood glucose.

DBI may also enhance the peripheral uptake of glucose. Beckman (123) has suggested that DBI enhances glucose uptake by muscle but not by adipose tissue in vivo. DBI also appears to potentiate the effects of insulin and the sulfonylureas since the
hypoglycemic actions of these compounds given together are greater than the sum of their individual hypoglycemic effects.

Further work, especially with human subjects, is necessary before the mechanism of DBI action can be completely understood.
CHAPTER VI

SUMMARY

Factors affecting hepatic synthesis and release of glucose were investigated using isolated perfused rat and guinea pig livers. These livers were perfused via the portal vein by recycling oxygenated Krebs-Ringer-bicarbonate buffer containing human serum albumin, glucose, and varying concentrations of free fatty acids. A parallel perfusion circuit permitted instantaneous change to fresh perfusion medium. Livers from fed rats were perfused alternately with high and low free fatty acid media. During high free fatty acid perfusion glucose was released by these livers. There was little or no release during low free fatty acid perfusion. This glucose release was due to activation of phosphorylase. Addition of free fatty acids (octanoate or oleate) to the medium perfusing livers from fed rats caused almost a doubling of phosphorylase activity.

The effects of free fatty acids on glycogen breakdown have permitted expansion of the concept of a glucose-fatty acid cycle to include the liver as well as muscle and adipose tissue. According to this expanded cycle, free fatty acids tend to elevate blood glucose by decreasing peripheral glucose utilization and by
stimulating hepatic glycogenolysis. Elevated blood glucose levels tend to lower plasma free fatty acids by enhancing reesterification and by triggering the release of insulin which further suppresses lipolysis. The glucose-fatty acid cycle provides a primitive mechanism whereby blood glucose and fatty acid levels are controlled independently of hormonal control.

In addition to stimulating glycogenolysis free fatty acids were shown to enhance gluconeogenesis. Free fatty acids enhanced the conversion of lactate and pyruvate to glucose but had no effect on gluconeogenesis from glycerol. There was a very slight enhancement of gluconeogenesis from oxaloacetate. However, this was only a fraction of that seen with lactate and pyruvate. It was therefore suggested that free fatty acid metabolism enhanced gluconeogenesis by generating acetyl CoA. Acetyl CoA is an allosteric activator of pyruvic carboxylase, one of the key gluconeogenic enzymes. This enzyme catalyzes the conversion of pyruvate to oxaloacetate. Thus it is involved in gluconeogenesis from lactate and pyruvate but not from oxaloacetate or glycerol.

The mechanism of action of the oral hypoglycemic compound phenformin (DBI) was also investigated. Low concentrations of this drug (10 mg.%) had no effect on glycogenolysis or gluconeogenesis in isolated perfused rat livers. High concentrations of DBI (50 mg.%) caused glycogenolysis in livers from fed rats whereas there was no effect on gluconeogenesis. It was subsequently
discovered that DBI had no hypoglycemic effect on rats in vivo. DBI did, however, cause guinea pigs to become hypoglycemic. Studies with isolated perfused guinea pig livers showed that DBI concentrations as low as 2 mg.% inhibited gluconeogenesis from lactate, pyruvate, oxaloacetate, and glycerol. Since DBI has been shown to interfere with the production of ATP, liver ATP levels were determined. It was found that DBI caused marked decreases in guinea pig liver ATP both in vivo and in vitro whereas it had no effect on rat liver ATP. It was therefore postulated that DBI inhibits gluconeogenesis by decreasing ATP concentrations. ATP provides the necessary energy for gluconeogenesis. In addition, decreased ATP concentrations are accompanied by increases in 5'AMP, which is an inhibitor of fructose-1,6-diphosphatase. Fructose-1,6-diphosphatase is a key enzyme involved in gluconeogenesis from lactate, pyruvate, oxaloacetate, and glycerol. Inhibition of this enzyme by 5'AMP would inhibit gluconeogenesis from all four of these substrates and fructose-1,6-diphosphate would be expected to accumulate. Perfusion of guinea pig livers with DBI did cause a slight accumulation of fructose-1,6-diphosphate. Thus the hypoglycemic action of DBI in guinea pigs can be explained by its interference with ATP generation.
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