THE EFFECT OF ACETOACETATE, ORGANOSULFUR COMPOUNDS AND HORMONES ON THE ACTIVITY OF THE LIVER MITOCHONDRIA

by

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I. INTRODUCTION

The biochemical basis for the metabolic disorder of cattle and sheep, referred to as ketosis, acetonemia or pregnancy disease, remains obscure.

Ketosis of cattle and sheep has been under extensive investigation for at least 30 years. To date many attractive theories have been advanced in attempts to understand the nature of the disturbance. However, these hypotheses, singly or in combination, fail to adequately explain the biochemical sequelae which terminate in this condition, or to clarify the factors which render animals predisposed to the malady.

The economic importance of this problem is illustrated by the report of Dye and Dougherty (1) in which it was estimated that over a million cases of ketosis occurred in 1950, among the 25,000,000 milk cows in the United States. This is an incidence of approximately four percent. Due to the monetary loss incurred through lowered milk production and premature removal of the ketotic cows from the herd, numerous therapeutic measures have been devised in an attempt to prevent and/or cure ketosis. Unfortunately, since our knowledge persuant to the biochemical aspects of the problem are rudimentary, prophylaxis and therapy are on the whole inadequate.

The primary goal of the investigations reported herein was to obtain information, which might be useful in the elucidation of the etiology of this systemic derangement.

II REVIEW OF THE LITERATURE

A. Biochemical and Histological Basis of Bovine Ketosis

This review will tend to be lengthy because of the extensive literature that has developed and the many interrelated facets of this problem. An attempt will be made to correlate this mass of information, in preparation for the experimental portion of this study. Some phases of this review may appear superfluous; however, they are essential for the understanding of this complex problem.

Ketosis of dairy cattle usually occurs after parturition and is rarely a problem in the pre-parturient cow. It is generally conceded to occur most frequently in heavily lactating animals either immediately following calving, in the so-called parturient milk fever type (2, 4, 5) or from 10 days to 2 months after term in the non-parturient digestive disorder (2, 3, 4, 6, 7). It has been reported by Roepke (8) that ketonuria is present in numerous cattle throughout gestation but few actually exhibit the classical symptoms of acetonemia. This corroborates the original observation by Sampson and Hayden (9).

Outward clinical symptoms of ketosis as stated in numerous papers (2,5) include a sudden loss of appetite, a rapid decline in body weight and a marked decrease in milk flow. It is generally accepted that the condition of ketosis in cattle and sheep is accompanied by a characteristic hypoglycemia and hyperketonemia (8, 10, 11). Likewise, low levels of liver glycogen and fatty infiltration of this organ are commonly encountered (11, 12). Recently another biochemical parameter has been added to the symptomatology of the ketotic syndrome. Bach and Hibbitt (13) have shown

that the levels of plasma pyruvate and α-ketoglutarate were markedly depressed in bovine ketosis. It was suggested that a possible block in the Krebs cycle occurs; more specifically in a reaction (or reactions) involving the conversion of pyruvate and α-ketoglutarate to citrate and succinate, respectively. This interesting work and the subsequent reports by these investigators (14, 15) have provided the impetus for the conduct of the experiments to be later expounded.

The possibility that ketosis in some way is connected to endocrine function has long been a basic precept. Some studies measuring the level of various circulating hormones, as well as some histopathological work, has been reported. Attention to this now appears germane. Shaw et al (16) were the first to note an adrenal involvement in bovine ketosis. The level of blood cholesterol was found to be depressed, while marked fatty infiltration and partial degeneration of the adrenal cortex were evident. Another point of interest was the relatively low level of adrenal ascorbic acid in afflicted animals (16, 17). Cystic degeneration, atrophy and fatty infiltration of the anterior pituitary gland of acetonemic cows have also been reported (18). From the above evidence it has been suggested that bovine ketosis involves a malfunction of the pituitary-adrenal system (18). Histopathological examinations of the pituitary and adrenal glands of ketotic cows support this view. The acetonemia syndrome has been classified as an adrenocortical insufficiency brought about by the acute stress of parturition and lactation. In support of this hypothesis considerable effort has been expended in the study of the blood eosinophil levels. A decrease in the number of circulating eosinophils has been observed by various investigators (20, 23) during the

parturient period, when the level of adrenal secretion was presumably increased by stress. However eosinophil counts of ketotic cows have been reported (21, 22) to be either normal or slightly augmented. The higher level during ketosis has been attributed to a hypofunction of the pituitary-adrenal axis. The relative function of the adrenal system appears to be centrally important in understanding the metabolic dysfunction, therefore the secretory activity of this gland has come under investigation. Puntriano (24) noted that levels of urinary glucocorticoids in pregnant cows were increased, while those in ketotics were decreased, when compared to normal animals. Querido et al (25) have stated that the levels of blood 17-hydroxycorticosteroids is the best criterion of adrenal cortical function. Robertson and Mixner (26) have measured the total free 17-hydroxycorticosteroids in blood and found them to be significantly elevated in non-lactating cows, in comparison with normal lactating, non-pregnant animals. This is in agreement with the observations by Gemzell (29) that the plasma level of 17-hydroxycorticosteroids are elevated during human pregnancy. A subsequent study by Robertson and coinvestigators (27) on ketotic cows, and by Robertson et al (28) on cattle subjected to stress situations other than ketosis are of interest. The levels of total 17-hydroxycorticosteroids were found to be augmented significantly in animals with ketosis, while the levels of serum protein bound iodine (P. B. I.) were depressed (27). In other stressful situations (28) the level of 17-hydroxycorticosteroids was also elevated; however, the serum P. B. I. was not significantly lowered. These results imply a thyroidal involvement in ketosis, and the data have been interpreted to

mean that this malady may be mediated through hypothyroidism (27). The level of plasma 17-hydroxycorticosteroids in bovine pregnancy and parturition have also been determined by Brush (30), who concurs with the original observation of Robertson and Mixner (26). It should be recalled that the determination of 17-hydroxycorticosteroids is a measure of cortisone, 17-hydroxycorticosterone and desoxycortisone and may in reality not be a valid parameter of adrenal cortical function. Other glucocorticoids, especially corticosterone, have been isolated from normal cow blood (31).

The depressed level of P. B. I. as noted in ketotic cattle (27), may be of paramount importance in the etiology of ketosis. It may therefore be of value to survey this facet of the literature. The data of Lewis and Ralston (32) suggests a decline in thyroidal activity post partum in cattle as measured by P. B. I. The P. B. I. level was depressed on the day of calving. Williams and coworkers (33) noted that adrenaline, typhoid vaccine and trauma depressed the rate of I^{131} uptake by the thyroid. Fasting has been shown by Bondy and Hagewood to decrease P. B. I. in rats (34) and by Mixner _et al_ (35) in calves. Blincoe and Brody (36) reported that starvation in cows produces a marked decline in thyroid gland I^{131} uptake. Further work on the occurrence of adrenal-thyroid interaction has been provided by Levin and Daughaday (37), who demonstrated that there is a slow rate of disappearance of exogenous hydrocortisone from the blood of myxedematous humans, indicating a decreased rate of steroid degeneration. Hill _et al_ (38) have observed that many myxedematous patients fail to give a normal adrenocortical response after administration of ACTH. In addition both ACTH and cortisone decreases the I^{131} uptake gradient and the level of P. B. I. in normal subjects.

Information concerning the activity of insulin in cattle during various physiological states, including ketosis, has recently become available. Cunningham (39) reported that the plasma insulin levels were significantly depressed after parturition. They were also very low in ketotic cattle and in sheep with pregnancy toxemia. Calhoun and coworkers (40) also observed a dimunition of serum insulin-like activity in the ketotic as well as in the fasting bovine. The true significance of hormonal changes in ketosis remains obscure. However, they may regulate homeostasis, in light of the report of Engel (41), who noted that increased 17-hydroxycorticosteroid elaboration resulted in accelerated protein catabolism through gluconeogenesis. Reece (42) and Smith and Dastur (43) observed that thyroprotein or thyroxine was beneficial in ketosis. This may be a reflection of an increased blood sugar (43). Information persuant to the metabolism and enzymatic efficacy in cattle and sheep suffering from acetonemia is extremely limited. Sauer, Dickson and Hoyt (44) have stated that the oxygen uptake of liver homogenates from ketotic cows was markedly depressed. Gallagher (45) observed normal respiration of liver and brain mitochondria isolated from sheep with pregnancy toxemia. Liver mitochondria were not able to oxidize octanoate and palmitate. This seems rather incongruous with regard to the postulated inhibition of the tricarboxylic acid cycle by Bach and Hibbitt (13). The work of Tombropoulos and Kleiber (46) indicates that in bovine ketosis there is a decrease in the hexose monophosphate shunt and the decrease seems to be correlated with impaired lipogenesis.

B. Therapeutic Measures as Related to Bovine Ketosis

A number of effective therapeutic measures have been proposed for the treatment of ketosis and are usually based upon the most outstanding symptoms of the disorder; namely hyperketonemia and hypoglycemia.

Intravenous infusion of glucose (47, 48) appears to be effective since it increases the level of circulating glucose, a metabolite known to be in short supply during ketosis. Administration of glucagon, the hyperglycemic principle secreted by the alpha cells of the pancreas, caused an increase in the blood sugar with a concomitant dimunition of ketone bodies in the blood of ketotic animals (49, 50). Administration of adrenal cortical extract (51) of cortisone (52), of hydrocortisone (53), of ACTH (54), of prednisone (55), of prednisolone (56) and of 11-ketoprogesterone (57), have all proven effective therapeutic agents. It may be well to recall the previously-mentioned report of Engel (41) who noted enhanced gluconeogenesis subsequent to increased 17-hydroxycorticosteroid secretion. Gavosto et al (58) noted that in cortisone-treated rats, the level of glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase of liver was markedly increased. The effect of various steroids upon the activity of these enzyme systems has been confirmed (59, 61). In a series of papers, Schultz (62, 64) noted that sodium propionate was effective in prevention and treatment of ketosis. It has been conclusively established (65, 71) that propionate can be converted to succinate and then used in the Krebs cycle.

Johnson (72) has postulated that bovine ketosis may be due to a shortage of oxalacetic acid, and that the lack of this essential four carbon intermediate allows acetyl coenzyme A to pile up, ultimately resulting in the disorder. Reber and Schoettle (73) have administered oxaloacetic

acid orally to sheep with pregnancy toxemia. This treatment was effective in causing remission of the hypoglycemia and hyperketonemia.

The most recent prophylaxis is that of cysteamine and/or fumarate as reported by Bach and Hibbitt (14,15). They (14) propose that ketosis may be due to reduced availability of coenzyme A. This idea is supported by the work of Peeters et al (74) who reported a diminished ability to acetylate sulfanilamide in ketotic cows. Gallagher (45) demonstrated a subnormal level of coenzyme A in the livers of ketotic sheep. Lynen (75) has reported that cysteamine has coenzyme activity. Administration of cysteamine (14, 15) results in remission of ketosis preceded by a drop in the abnormally elevated blood pyruvate and α-ketoglutarate levels.

It seems evident that all of the effective therapeutic measures mentioned may conceivably exert their beneficial effects by either increasing the already raised levels of pyruvate, or by removing a postulated block in the Krebs cycle. Indeed various therapeutic hormones such as hydrocortisone (76, 77), prednisolone (76) and thyroxine (78) all cause increased blood pyruvate in the normal animal. This augmented substrate concentration could possibly reverse the proposed inhibition of the Krebs cycle by the Law of Mass Action.

C. Ketogenesis and Ketolysis

It is generally agreed that the liver is the chief, if not the sole, source of the ketone bodies (79, 84). Conversely, ketolysis is mainly an extrahepatic function (79, 80, 82, 89). The mechanism of β-oxidation whereby fatty acids are degraded, was first proposed by Knoop (90). The fatty acid oxidase system, which utilizes long chain fatty acids, converting them to acetoacetate, is located in the mitochondria (91, 92).

It has been observed *in vivo* (93, 94) and *in vitro* (95, 96) that the β-oxidation of even numbered long chain fatty acids leads to acetoacetate formation, while the oxidation of odd chain substrates does not result in ketone body accumulation. Lipmann (97) discovered coenzyme A and recognized its key role in acetate metabolism. It was subsequently observed by Lynen and Reichert (98) that the active form not only of acetate but also that of higher fatty acids were acyl thioesters of Coenzyme A. The complete definition of the individual enzymatic reactions involved in β-oxidation was finally accomplished some 50 years after the theory was originally proposed. The sequential order of the fatty acid oxidation cycle enzymes has been outlined by Lynen (99).

It may now be propitious to dwell upon the enzymatic formation and utilization of acetoacetate in order to gain additional understanding of the ketosis problem. MacKay (100) proposed that acetoacetate is produced in the liver by the condensation of two carbon moieties derived from the oxidation of fatty acids. This postulate has been proven and further elucidated by numerous workers (101, 106). Specifically (106), 2 Acetyl CoA $\longrightarrow$ Acetoacetyl CoA + CoA. A deacylase (thioesterase) present in bovine liver has the capacity to cleave the thiol ester according to the reaction: Acetoacetyl CoA + H_2O Acetoacetate + CoA. This reaction is non reversible (106). Additional evidence has been presented in support of the contention that the above pathway accounts for the bulk of acetoacetate formed from acetoacetyl CoA (107, 112). Specifically, acetoacetate formation proceeds unimpaired in the presence of iodoacetamide. This compound has been reported to inhibit acetoacetate formation from β-hydroxy β-methyl glutaryl CoA (HMG-CoA). In addition, the results of labeling experiments appear to support the view that the HMG-CoA pathway is nearly inoperative in mitochondria.

However, another body of evidence with regard to the formation of acetoacetate from acetyl-CoA has built up around the observation of Lynen _et_ _al_. (113) that HMG-CoA is an intermediate in the biosynthesis of acetoacetate. The mechanism involved requires two enzymes and is thought to occur by the sequence: 1. acetyl CoA + Acetoacetyl-CoA + $H_2O \longrightarrow$ HMG-CoA + CoA SH, 2. HMG-CoA $\longrightarrow$ acetoacetate + acetyl-CoA.

Additional indications in support of this are provided by continued investigations (114-116). Sauer (116) proposed that both systems for forming acetoacetate were present and operative. Caldwell and Drummond (115) have concluded from their recent study that the HMG-CoA pathway accounts for most if not all of the acetoacetate formed by liver preparations. At present the controversy remains unresolved.

McCann (117) has reported that the mitochondria of kidney, heart, brain, and muscle can oxidize acetoacetic acid. Liver mitochondria do not have this ability. The enzyme acetoacetic thiophorase, which acylates the free acid, has been shown to be ubiquitous for all tissues with the exception of liver (117-120).

Another enzyme system which has been implicated in the production of acetoacetyl-CoA is the acetoacetic thiokinase, which is operative according to the scheme: Acetoacetate + ATP + CoA SH $\longrightarrow$ Acetoacetyl-CoA + AMP + PP_i (117-119). This system is proported to be omnipresent with the exclusion of the liver. Thus,

we are confronted with the enigma whereby the liver can very actively produce acetoacetate from acetoacetyl-CoA, but is relatively incompetent in producing the thioester from the free acid.

The requirement of succinate for acetoacetate synthesis from acetyl-CoA in extracts of heart and kidney (106, 121) led to the demonstration of the enzyme CoA transferase (122). The reaction occurs by the following mechanism.

Succinyl-CoA + Acetoacetate ⟶ Acetoacetyl-CoA + Succinate

However, this reaction is not applicable to liver where CoA transferase could not be demonstrated (106, 122).

Krebs (123) has recognized that liver does not actively utilize free acetoacetic acid, but that small amounts disappear resulting in the formation of free β-hydroxybutyric acid. The removal of acetoacetate by the liver was accelerated by the *in vitro* addition of various hydrogen donors such as α-ketoglutarate, glutamate, pyruvate, citrate, malate, and succinate. This enhancement of aerobic removal of acetoacetate by Krebs cycle intermediates and inhibition by malonate has been extensively studied (124-132). These studies are in concord with that of Kennedy and Lehninger (91), who concluded that fatty acid oxidation is enhanced by the presence of a Krebs cycle intermediate, acting as a primer. Subsequent studies (133) have demonstrated that DPNH also is an effective primer. In the presence of DPNH long chain fatty acids

are converted mainly to acetoacetate, but in the presence of oxaloacetate or other cyclic intermediates, the acetate derived is shunted through the Krebs cycle. More recently, Devlin and Bedell (134, 135) have observed that DPNH oxidation by mitochondria is stimulated by the addition of acetoacetate or by D(-) β-hydroxybutyrate. The results of Krebs (123-131) and of others (132-135) may be important in the hypothesized inhibition of the Krebs cycle (13) leading to ketosis.

D. Conditions Leading to Increased Ketogenesis

It is now firmly established that the administration of growth hormone results in the mobilization of nonesterified or free fatty acids from adipose tissue. This is evidenced by increased plasma FFA concentration (136-138) and increased release of FFA from adipose tissue *in vitro* (139).

Since the initial observation by Burn and Ling (140) that crude pituitary extract enhanced ketonuria, considerable effort has been directed to the study of the *in vivo* and *in vitro* effects of anterior pituitary hormones on ketone metabolism. That these anterior pituitary extracts, initially crude, but then more purified to include growth hormone, adrenocorticotropic, and thyrotropic hormones, enhance ketone body formation is well documented. To cite but a few of the myriad references will suffice. Shipley (141) reported augmented

ketogenesis by surviving liver slices when crude pituitary extract was added *in vitro*. Lotspeich (142) observed that liver slices from female rats previously injected with purified growth hormone produced considerably greater quantities of acetoacetate than did slices from normal female rats. Thyrotropin (143, 144) and adrenocorticotropin (144, 145) cause increased levels of blood ketones in the rat. ACTH (145) appears to exert its effect by an extra-adrenal action, since adrenalectomy does not diminish the response. It is conceivable that purified growth hormone will be classified as non-ketogenic (143) since the most purified preparations fail to elicit ketogenic activity.

Fasting or starvation can cause ketosis in a wide variety of species (146-148), presumably triggered through lack of metabolizeable substrates.

Ketosis is also manifest in diabetes mellitus. It has also been frequently noted in alloxan-treated animals. Moriwaki *et al*. (149) have reported that diabetic humans manifesting disturbed acetylation excreted more ketone bodies than other diabetics. Beatty and coworkers (150) have observed decreased utilization of acetoacetate by muscle of alloxan diabetic rats. This supports the early postulation of Shaffer (151) who proposed that a decrease in peripheral utilization of ketone bodies is a contributing factor in diabetic ketosis.

Burn and Ling (140) noted that rats in the terminal stage of pregnancy excreted considerable quantities of ketone bodies in the urine. Antognetti and Scopianaro (151) were unable to find constant hyperketonemia during the course of human pregnancy. However, a humoral hyperketogenic factor was found during the final phase of pregnancy and particularly during labor, which caused ketogenesis in rats. Scow and co-investigators (152) reported that marked ketosis occurred in fasted pregnant rats, but only during the second half of the gestation period. It is interesting to recall that during ketosis in the bovine the level of circulating 17-hydroxycorticosteroids is increased (27). The plasma level of 17-hydroxycorticosteroids is also increased in human pregnancy (23). Plasma levels of 17-hydroxycorticosteroids are likewise increased in pregnancy at the terminal third of gestation in the rat (153-155). In severe insulin deficiency, in which carbohydrate utilization is impaired, cortisone is strongly ketogenic (152). Bergman and Sellers (156) have succeeded in producing ketosis in pregnant guinea pigs by subjecting them to a three-day fast during the terminal two weeks of gestation.

A more profound ketonuria has been found to occur in female rats than in males exposed to ether anesthesia (157). In rats anesthetized with Nembutal, smaller doses of ACTH were effective in producing ketonemia than in unanesthetized animals (158).

Khanade and Nath (159) have shown that liver slices from thiamine-deficient rats produce greater amounts of acetoacetate, while ketone utilization in deficient animals by kidney and diaphragm is depressed.

E. Metabolic Effects of Ketone Bodies

Acetoacetate administration to normal intact animals or normal isolated tissues produced considerable detrimental effects, which all point to the premise that it is a toxic substance.

Nath and Chakrabarti (160) noted that the long-term administration of either acetoacetate or β-hydroxybutyrate to rabbits produce a condition resembling riboflavin and nicotinic acid deficiency. A vitamin B_1 depletion in blood and urine could be produced by long-term acetoacetate or β-hydroxybutyrate administration with production of a simultaneous increase in blood and urine pyruvate (161). Blood lactate could be increased and ascorbic acid depressed by acetoacetate injection (162). Reduced-glutathione levels in blood were depressed by acetoacetate administration (163-165). Illing et al. (166) have found a significant reduction in blood reduced-glutathione levels in diabetics with ketosis. Milla and Capraro (167) and Tidwell and Axelrod (168) report that injection of acetoacetate results in hypoglycemia. Glycogen synthesis by rat diaphragm (169, 170) in vitro was depressed,

after addition of acetoacetate. A histopathological effect of acetoacetate administration has been reported by Nayadu *et al.* (171) who cites hypertrophy of the Islets of Langerhans of the pancreas.

F. Conditions Leading to Diminished Ketogenesis

The well-known antiketogenic effect of glucose upon the level of circulating ketone bodies (172-174) has been amply documented. Glucose has also been shown by Neptune *et al.* (175) to increase the utilization of ketone bodies *in vitro* by the excised rat diaphragm. The effectiveness of glucose in bovine ketosis has already been referred to (47, 48).

Terner (176) has reported that lactate was antiketogenic and proposed that this substrate and glucose may exert their effect by virtue of being DPNH generators. Sorbitol, which can lead to the formation of fructose and DPNH, has been noted to be a potent antiketogenic factor *in vitro* (177, 178).

Insulin, which has been shown to be decreased in the blood of ketotic cows (39, 40), has been implicated in ketogenesis. An early report by Campbell (179) concerns the effectiveness of insulin in reducing the high ketone level of the blood and urine in humans with diabetic ketosis. The antithetic action of insulin on ketogenesis by liver slices *in vitro* has been reported (89, 180). Somogyi (181) has proposed that insulin exerts two opposite effects upon ketonemia.

For sometime after injection, insulin causes a decrease, but after protracted states of hypoglycemia, it can effect a rise in ketone level. Initially insulin is thought to inhibit glycogenolysis, but during the second hypoglycemic phase, its action appears to cause stimulation of glycogenolysis.

Cortisone as well as other glucocorticoids, steroids and ACTH (51-57) have been widely used as therapeutic measures in bovine ketosis. Administration of cortisone can diminish the ketonemia in rats caused by fasting, cold exposure and sodium fluoroacetate administration (182-185). The possible mode of action of these substances relative to the activity of transaminase systems has already been discussed (58-61).

It has been suggested that a lack of (72), and administration of (73) oxaloacetate is respectively causative and curative in bovine and ovine ketosis. The studies on tricarboxylic acid cycle intermediate enhancement on removal of acetoacetate in vitro may have a bearing on the oxaloacetate debt (123-133). Beatty and West (186) have reported that various precursors of oxaloacetic acid were able to decrease the total urinary ketone bodies in rats made ketotic with butyrate. Further studies (187) on the ketotic alloxan diabetic rat showed that succinate, especially in conjunction with insulin was markedly antiketogenic. The effects of insulin and succinate together were more than additive. Beatty and West (188) have proposed that

insulin may be involved in metabolism at the level of the tricarboxylic acid cycle. Beatty et al. (189) noted that liver slices from normal fasted rats produce less ketone bodies upon the addition of succinate and fumarate. Ketone body production of liver slices obtained from fed diabetic rats were reduced by either fumarate or oxaloacetate addition.

It is of interest to note the effect of tolbutamide, an oral hypoglycemic agent upon ketosis. Tolbutamide has been reported to decrease diabetic ketonemia (190, 191). Dimunition of fasting ketosis in the rat upon administration of this drug has also been noted (192). Its action may possibly be attributed to induced insulin-like activity (193), observed after tolbutamide administration.

Liver slices from pantothenic acid-deficient rats had diminished oxygen uptake and ketone body production from octanoate in comparison to normal (194).

G. Utilization of Pyruvate and α-ketoglutarate Via the Krebs Cycle

In view of the proposed Krebs cycle block supposedly occurring in bovine ketosis (13-15), which is thought to cause increased blood pyruvate and α-ketoglutarate levels, a comprehensive study of the relevant literature is mandatory.

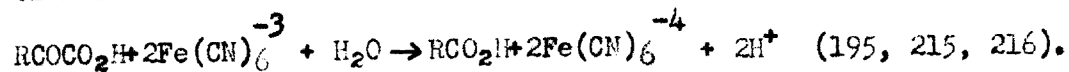
It has been recognized that the oxidative decarboxylation of pyruvate and α-ketoglutarate via the Krebs cycle is not carried out by one enzyme in each case, but by a so-called enzyme complex (195, 196). The capacity to utilize either substrate is designated by

prefixing the name of the substrate to either oxidase or dehydrogenase. The individual enzymes of the complexes have been purified, isolated and recombined to form the active complex (196, 198). Koike et al (195) reported that the molecular weights of the pyruvic and the α-ketoglutarate complexes are respectively 4.8 and 2.4 million.

Thiamine pyrophosphate was implicated in pyruvate utilization by Coxon and Peters (199) who observed enhanced citrate formation upon addition of thiamine pyrophosphate (TPP). Franken and Stapert (200) reported that pyruvate oxidation was impaired in thiamine deficiency. TPP reactivated the ferricyanide system, but lipothiamide pyrophosphate was necessary for complete reactivation of the oxygen-dependent electron transport scheme. The pyruvic dehydrogenase complex loses thiamine as well as some of its lipoic acid in thiamine deficiency. The possibility of a diphosphothiamine acetaldehyde derivative in pyruvate metabolism was first put forward by Lynen and Reichert (98). Breslow (201), and Krampitz et al (202) hypothesized that the aldehyde moiety in the theoretical "acetaldehyde thiamine pyrophosphate" is linked to the 2 position of the thiazole ring by an α-hydroxyethyl group forming α-hydroxyethyl thiamine pyrophosphate (HETPP). Experimental evidence in support of this suggestion has been obtained (203, 205). The enzymatic formation of HETPP has been demonstrated by Holzer and Beaucamp (204), as an intermediate in the decarboxylation of pyruvate. Carlson and Brown (206) have found HETPP to be naturally occurring in microorganisms and have also demonstrated its enzymatic formation. Labeled HETPP has been isolated from yeast mitochondria after incubation with radioactive pyruvate (207). Goedde et al (208) have demonstrated that HETPP, or

active acetaldehyde is an intermediate in the oxidation of pyruvate to acetyl CoA. Incubation of purified pyruvic oxidase from pig heart with TPP and pyruvate in the absence of CoA and DPN results in the accumulation of HETPP (209, 210). Presumably, active succinaldehyde is the intermediate in α-ketoglutarate oxidative decarboxylation.

Enzyme systems which catalyze a coenzyme A and diphosphopyridine nucleotide-dependent oxidative decarboxylation of pyruvate and ketoglutarate by the reaction $RCOCO_2H + CoASH + DPN \longrightarrow RCO\text{-}S\text{-}CoA + CO_2 + DPNH + H^+$, have been isolated from mammalian (211, 213) and bacterial (195, 214) cells. Ferricyanide can serve as the electron acceptor for either substrate in the above reaction according to the scheme:



$RCOCO_2H + 2Fe(CN)_6^{-3} + H_2O \rightarrow RCO_2H + 2Fe(CN)_6^{-4} + 2H^+$ (195, 215, 216).

It has been stated that the ferricyanide reaction is the most rapid α-keto acid-mediated system (215, 216). The ferricyanide reaction proceeds in the absence of added DPN^+ or CoA (212, 216, 217). The non-ferricyanide system has been shown to require at least four cofactors: DPN^+, CoA, Mg^{++} and TPP (213, 214). It is of interest to note that α-lipoic acid is essential for linking ferricyanide to _S. fecalis_ pyruvic apodehydrogenase (217). Both enzyme complexes contain tightly bound, non dialyzeable α-lipoic acid or some derivative thereof (212, 218). TPP is also tightly bound and non dialyzeable (212). Gunsalus and coinvestigators (219) have reported that α-lipoic acid is essential for the functioning of pyruvic oxidase. Seaman and Naschke (220) have removed α-lipoic acid from both enzyme complexes which results in their inactivation. Activity was restored by adding back catalytic amounts of lipoic acid to the apoenzymes. Reed and DeBusk (221) have reported

an enzyme, lipoic conjugase, which can synthesize lipothiamide. Lipothiamide pyrophosphate, LTPP, has been found to be more active than lipoic acid and is postulated to be the active coenzyme (222). Further evidence for LTPP as the active form of the coenzyme has been provided (223). Searls and Sanadi (224) and Massey (225) have fractionated the α-ketoglutarate complex into two parts. The colorless fraction, that containing all of the TPP and α-lipoic acid was found to catalyze the oxidation of α-ketoglutarate when ferricyanide was the electron acceptor.

Another enzyme of the two complexes, called dihydrolipoic transacetylase or lipoic reductase-transacetylase has been isolated and studied (226, 227). Its mechanism of action is envisioned as:

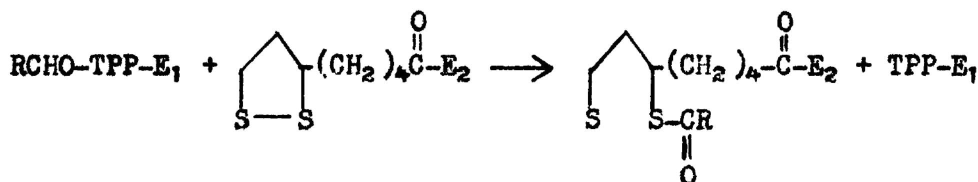
$$CH_3CO\text{-}SCoA + Lip(SH)_2 \longrightarrow CH_3COS\ Lip\ SH + CoA\ SH$$

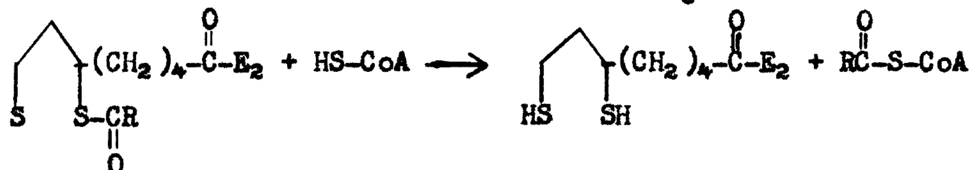
Considerable effort has been expended on the study of another enzyme of the complexes, known as dihydrothioctyl (dihydrolipoyl) dehydrogenase. The enzyme catalyzes the following reactions (228):

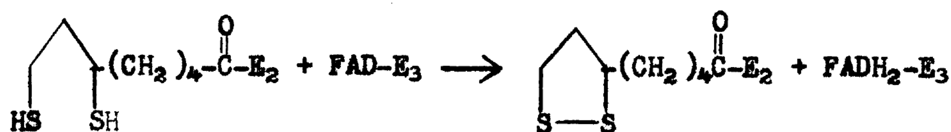
$$DPNH + H^+ + T(S)_2 \longrightarrow DPN^+ + T(SH)_2 \text{ or}$$

$$DPNH + H^+ + Dye \longrightarrow DPN + Dye\ H_2$$

This enzyme has been shown to be a flavoprotein (224, 229-231), closely related to Straub's diaphorase (232). That diaphorase is identical to lipoyl dehydrogenase has been the subject of considerable study by Massey (233-236). Searls and Sanadi (237) are in accord with Massey (233-236). However, Zeigler, Green and Doeg (250) are not in agreement that lipoyl dehydrogenase and Straub diaphorase are synonymous. Based upon the evidence available it is believed that the CoA, DPN-dependent oxidative decarboxylation of pyruvate and α-ketoglutarate proceeds via the scheme (238):







The sulfhydryl nature of the pyruvic and α-ketoglutaric oxidase complexes have been alluded to for a considerable time. Barron and Singer (293) enhanced the rate of pyruvic acid oxidation by liver homogenates by the addition of reduced glutathione. Sulfhydryl reagents inhibited both the oxidation of pyruvate and α-ketoglutarate. This inhibition was reversed by reduced glutathione addition. The effect of SH reagent inhibition on the complexes has been widely documented. Massey (240) has reported para chloromercuribenzoate, PCMB, inhibition of lipoyl dehydrogenase, a component enzyme of the complex systems. Arsenite has been reported by Krebs (241) to be an inhibitor of the oxidation of α-keto acids. This inhibition by arsenite has been shown to be due to combination with thiol groups (242). Stocken et al (234) observed that British Anti Lewisite (BAL), a dithiol, could reactivate inhibited pyruvic oxidase. Arsenite inhibition is poorly reactivated by monothiols

but well reactivated by dithiols (244,245). Searls and Sanadi (228) have shown that dihydrothioctyl dehydrogenase forms a complex with arsenite or cadmium and is inactivated. Reactivation is nearly complete with a dithiol (BAL); however a monothiol (cysteine) was less effective even at increased levels. Lipoamide dehydrogenase has been found by Veeger and Massey (246) to be inhibited by trace minerals, especially by Cu^{2+}. Other divalent cations were reported to inhibit the pyruvic and α-ketoglutarate complexes by forming chelates with dihydrolipoic acid (247, 248).

An interesting report by Fahmy et al (249), on the inactivation of the pyruvate and α-ketoglutarate complexes by thiopentone should be kept in mind. Partial reactivation was achieved by addition of either TPP or α-lipoic acid and complete reversal was attained when they were both added.

H. Conditions Affecting Pyruvate and α-Ketoglutarate Metabolism

Attention to the increased blood pyruvate and α-ketoglutarate in bovine ketosis has already been emphasized (13-15).

Human diabetes mellitus is another metabolic disorder in which increased levels of blood pyruvate have been reported (251-254). However this finding is not universal (255-257), but seems to be associated with the severe uncontrolled aberration (252).

Increased blood pyruvate levels have also been reported in humans suffering from Cushing's Syndrome (258, 259), a disorder characterized by generalized adrenal cortex hyperplasia with attendant hypersecretion of corticoids.

Augmented blood pyruvate has also been noted in cirrhosis of the liver (260) in thiamine deficiency (261), in alloxan induced diabetes (262), in poisoning with arsenite and lewisite (263), and by administration of oral antidiabetic drugs (264).

Considerable evidence for an effect of hormones on the level of α-keto acids in the blood has accumulated. Stadie and coworkers (265) have observed an increase in the utilization of pyruvate by pigeon breast muscle mince, following insulin addition. Insulin has also proven effective *in vivo* in lowering blood pyruvate levels. Koeppe *et al* (266) found that insulin could decrease the level of pyruvate in the alloxan diabetic rat. The metabolic efficiency of insulin in diminishing circulatory pyruvate has been widely expounded. Glucagon administration to normal humans results in an increase in blood pyruvate, diametrically opposed to that of insulin (267).

Treatment of humans with either cortisone or ACTH has been reported to induce an increase in blood pyruvate (259). The effect of cortisone (268, 269) and of ACTH (268) have been corroborated. The administration of cortisone appears to be without effect on circulatory α-ketoglutarate levels (269). Kerppola (270) has noted that mitochondrial oxidative phosphorylation of cortisone treated female rats was inhibited when pyruvate or α-ketoglutarate was the substrate. Cochran and Dubois (271) found that progesterone inhibited the oxidation of pyruvate and α-ketoglutarate.

Growth hormone treatment has resulted in the observation that pyruvate utilization is impaired in the intact STH treated dog (272).

Human growth hormone administration has been noted to increase the fasting blood pyruvate concentration in hypophysectomized patients (273).

Chronic thyroxine treatment has been reported to result in a higher level of circulating pyruvate (274, 275). Thyroidectomy was followed by a marked dimunition in pyruvate level (275).

Kyank (276) stated that after glucose or fructose infusion a greater rise in serum pyruvic acid was observed in late pregnant women compared to non pregnant controls.

In view of the increased pyruvate concentration seen in liver damage, Thompson et al (277) postulated a failure of lipoic acid containing enzyme systems. Shigeta et al (278) have noted a reduction in the serum lipoic acid level in hepatitis, liver cirrhosis, and in severe diabetes mellitus. Administration of lipoic acid to normal humans (279), or to hyperpyruvemic alloxan diabetic rats (280), has been reported to lower the α-keto acid content of the blood.

From the literature, it is apparent that a direct relationship exists between the metabolism of the α-keto acids and the ketone bodies. It is a well established fact that the perfused heart is actively able to utilize carbohydrate, and products of carbohydrate metabolism. Likewise, the heart muscle has been shown to be efficient in the oxidation of ketone bodies and fatty acids (281-283). Indeed, Hall (284) and Shipp et al (282) have reported that acetoacetate is preferentially utilized by this tissue, even in the presence of glucose. Newsholme and coinvestigators (285) have observed that the phosphofructokinase reaction, which catalyzes the conversion of fructose-6-phosphate to fructose 1, 6 diphosphate is inhibited in the normal perfused

rat heart by addition of acetoacetate, β-hydroxybutyrate, octanoate, butyrate and pyruvate. A subsequent study by Garland et al (286) on the isolated perfused normal rat myocardium, has demonstrated that pyruvate utilization is reduced by addition of either β-hydroxybutyrate, acetoacetate, or octanoate addition to the perfusate. Similar results have been obtained by Olsen (287) who has theorized a competition in the metabolism of these compounds, especially between acetoacetate and pyruvate on one hand, and fatty acids on the other.

I. Significance of Mitochondrial Swelling and Contraction

The phenomenon of mitochondrial swelling was initially observed by Raaflaub (288, 289). Since that time a considerable body of evidence has accumulated persuant to various swelling and contracting factors. As a number of these substances have been used in the work to be reported in this dissertation, it may be of value to discourse upon this effect.

Isolated mitochondria have been found to undergo swelling with the concomittant uptake of water by such diverse substances as ionic calcium (288, 289), zinc ions, phosphate and thyroxine (290), phlorizin (291), reduced glutathione (292), oxidized glutathione, and the disulfide hormones, oxytocin, vasopressin and insulin (293), by ferricyanide (294), by arsenite, BAL and cadmium ions (295), by the estrogenic hormones, diethylstilbesterol, hexesterol and dienesterol (296) as well as by cysteamine (297), and ferrous ions (298). Ergothioneine, thioglycolate, and thiolhistidine (297) as well as dihydrolipoic acid (292) have been found to be ineffective in inducing swelling of isolated mitochondria.

On the other hand, isolated mitochondria have been shown to undergo contraction with the concurrent extrusion of water (299, 300). This expulsion of fluid appears to be under the control of the so called "C factor", which has been isolated and purified (301). Reversal of swelling can be accomplished by the addition of ATP (299, 300) or magnesium ions (288). EDTA has been shown to be nearly universal in its protection against mitochondria distention (302).

Swelling has also been reported to occur in respiring mitochondria, especially upon succinate addition (288-290). This has resulted in the popular hypothesis that oxidative phosphorylation and mitochondrial volume changes are intimately related. It has been theorized that active electron transfer between substrates and oxygen is a prerequisite for inflation of isolated liver mitochondria (302). This view is further expounded in the review by Lehninger (303). Hunter et al (302) have also proposed that there may be two discrete types of swelling.

Aebi and Abelin (304) have reported that mitochondria from hyperthyroid subjects appeared to be swollen. Mitochondria isolated from hypothyroid rats swelled less when placed in hypotonic medium, while those inclusions obtained from hyperthyroid animals had enhanced swelling compared to normal (290). Magnesium low mitochondria, isolated from the heart and liver of magnesium-deficient rats were found to swell more rapidly (305). Mitochondrial particles isolated from the liver of essential fatty acid deficient rats were noted to be distended and also exhibited uncoupled oxidative phosphorylation (306). Malamud (307) stated that the cristae mitochondriales become fewer, shorter and less tightly pleated during induced swelling.

The concept that permeability of membranes of cellular particles are influenced by hormones is not new, but seems to have arisen from the work of Levine et al (308), who noted that permeation of sugars was affected by insulin. Krahl (309) has proposed that insulin combines with and contributes to the integrity of lipoprotein membranes wherever they occur. It is of relevance to note that four different disulfide hormones, namely insulin, vasopressin, and oxytocin (293) and growth hormone (310), are capable of causing mitochondrial protuberation. ACTH, a non disulfide containing hormone, is also effective in promoting swelling (310). A number of disulfide containing proteins which have no hormonal action have been observed to be ineffectual in causing swelling. Reference should be made to the interesting report of Fong et al (311), who suggested that a thiol-disulfide exchange reaction takes place between the disulfide hormone vasopressin, and sulfhydryl groups on the mitochondrial membrance surface. This results in the formation of a hormone receptor disulfide culminating in induced alteration of the tertiary structure of membrane proteins. Accordingly the resultant molecular sieve-like structure presumably allows for an increase in the flux of water as well as certain solutes. This view of essential mitochondrial surface sulfhydryl groups is supported by the investigations of Tapley (290) and Hunter et al (312) who reported that ionic mercury and copper, as well as paracloromercurybenzoate, (PCMB), and iodoacetate, all are effective swelling agents. These compounds share the common property of being able to combine with sulfhydryl groups. Thyroxine has been reported to be able to bind strongly with mitochondria and microsomes (313, 314). Since thyroxine has been observed to be a potent swelling factor (290),

it is not inconceivable that this swelling induced by this class of hormones may be an essential facet of their physiological action.

A variety of pertinent points have been discussed in this review, of which a few have been selected for consideration with regard to the ketosis problem. One may wonder if there is a metabolic block in the Krebs cycle *in vivo*, accounting for the increased levels of pyruvate and α-ketoglutarate, and if the increased level of ketone bodies could have an effect on the enzyme systems concerned with their utilization. The possibility that sulfhydryl compounds and hormones exert considerable effect upon the enzymes responsible for the oxidative decarboxylation of α-keto acids cannot be overlooked. In an attempt to gain further evidence with regard to these problems an investigation was conducted on the isolated liver mitochondria from normal and ketotic specimens. The results of this work follow.

III. THE INVESTIGATION

A. Materials and Methods

The experimental animals used in this study were adult female albino rats (175-250 grams) purchased locally,[1] and were of a Sprague-Dawley derived line. Female guinea pigs, of the English Shorthair breed were purchased from Rockland Farms,[2] and served both as subjects and as a colony for additional animals. The cattle were female registered Holsteins of the Virginia Agricultural Experiment Station herd.

Rats were fed a commercial diet[3] *ad libitum* and were individually housed in galvanized wire-bottomed cages. The guinea pigs received a commercial diet[4] *ad libitum* supplemented with alfalfa hay, and a solution of ascorbic acid dissolved in liquified sucrose. These animals were maintained in a specially constructed cage, with a solid-bottomed floor covered with wood shavings. On this regimen, no outward appearance of scurvy was noted during the course of these studies.

All chemicals used in this investigation were purchased from commercial sources, and utilized without further treatment. Materials that could not be bought were synthesized in accord with existing methods. Sodium acetoacetate was prepared by saponification of ethyl acetoacetate as outlined by Krebs and Eggleston (127). It was made

---

1. Dublin Laboratory Animals, Box 846, Dublin, Virginia
2. Rockland Farms, New City, New York.
3. Purina Lab-Chow
4. Pillsbury Best Rabbit Pellets

fresh before each experiment, and the concentration was determined by use of the method of Walker (315). D,L-dihydrolipoic acid was prepared by borohydride reduction of α-lipoic acid as reported by Drummond and Stern (316). It was prepared immediately before use and the concentration ascertained by the method of the aforementioned workers (316).

When measured, total blood ketones were determined by the method of Bakker and White (317), while blood glucose was carried out by the glucose oxidase assay (318). Blood was collected by decapitation in the small animal studies, while for cattle, it was drawn by puncture of the jugular vein. In all cases heparin was used as the anticoagulant.

Liver mitochondrial α-ketoglutarate and pyruvate activity were experimentally determined by use of the ferricyanide assay system of Gubler (319), with modifications. A brief description of the method as carried out in these studies now follows. The small animals were sacrificed by decapitation, and were exsanguinated. The livers were rapidly excised and placed in .25 M sucrose in vessels stored in cracked ice. Liver samples of cattle were obtained by the biopsy technique under light local anesthesia and treated similarly. A ten percent homogenate of the liver was prepared by addition of the requisite amount of cold .25 M sucrose to a pre-weighed quantity of liver. The tissue was coarsely minced with scissors, and was hand homogenized with the aid of a conical, loose-fitting, all glass, chilled Potter-Elvehjem tissue grinder. Liver mitochondria were separated by

differential centrifugation in the cold by a modification of the Schneider and Hogeboom method (320). Nuclei and cell debris were removed by centrifuging at 755xg for 10 minutes. The sediment was discarded, and the supernatant recentrifuged at 8,500xg for an additional 10 minutes. The preparation was defatted by pouring off the floating stratum, and by carefully wiping out the interior walls of the centrifuge tube. The fluffy layer was also removed by gentle shaking of the tube after the addition of a small quantity of cold .25M sucrose and subsequent decantation. The mitochondrial pellet was washed by resuspending it in cold .25M sucrose with the aid of a chilled Potter-Elvehjem homogenizer. This was followed by an additional centrifugation at 8,500xg for 10 minutes. The final mitochondrial preparation was obtained after decanting the supernatant and carefully resuspending the resultant pellet in cold .25M sucrose with the aid of a chilled hand driven Potter-Elvehjem. The final mitochondrial concentration represented a 1:5 dilution with respect to the original weight of fresh whole liver taken for homogenization. A 0.2 ml aliquot of this mitochondrial suspension was used for the assay of rat and guinea pig activity while .4 ml aliquots were necessary for the measurement of the enzyme level of cattle. The standard assay system conformed closely with that reported by Gubler (319). The complete reaction mixture contained potassium phosphate buffer 75 µM, pH 7.4; $MgSo_4$, 20 µM; sodium EDTA, 2 µM; sodium ATP, 6 µM; sodium pyruvate, 20 µM, or α-keto-glutarate, 40 µM; sucrose, 250 µM; 0.2 or 0.4 ml aliquot of

mitochondrial suspension; and demineralized water to make a total volume of 3.0 ml. Stock solutions of the organic constituents were prepared daily as required, and were carefully neutralized with dilute potassium hydroxide to pH 7.0 before use. The reaction tubes (including enzyme source) were prewarmed for three minutes at incubation temperature. The reaction was initiated by the timed addition of 0.7 ml of 0.00666 M potassium ferricyanide. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The tubes were centrifuged at room temperature, and the color density of the resultant supernatant was determined in the Evelyn colorimeter at 440 mμ. For rat liver mitochondria, the most convenient reaction temperature was found to be 25° C. With α-ketoglutarate and pyruvate as substrates the reaction times were respectively 25 and 50 minutes. For guinea pig liver mitochondria the reaction temperature was increased to 30° C and the reaction time was 50 minutes for both substrates. Bovine liver mitochondria could best be assayed at 37° C for 50 minutes with either substrate. Mitochondrial protein was determined by the Folin-Ciocalteau reaction in accordance with Sutherland _et al._ (321) as modified by Lowry _et al._ (322).

Ketosis in fasting late pregnant guinea pigs was induced in conformity with the report of Bergman and Sellers (156). A ketotic-like condition in the lactating bovine was induced by feeding preparturient cattle grain _ad libitum_. Two weeks post partum the animals were subjected to a six-day total fast.

Swelling of isolated mitochondria was studied utilizing the technique of Lehninger et al. (323), in which the mitochondria were isolated according to previously mentioned modifications as outlined in this section. In these studies the mitochondria derived from one gram of fresh liver were contained in one ml of 0.25M sucrose.

B. Results

The data to be reported in this section summarize experiments conducted on three species of animals, the rat, the guinea pig, and the lactating bovine. In all instances the subjects were mature females. These studies were initiated in the anticipation that the hyperpyruvecemia and hyperketoglutaremia noted in cattle in ketosis (13) might lead to the elucidation of a significant biochemical lesion.

It has been well established that the mitochondria represent the site of the tricarboxylic acid cycle enzymes. Therefore, an isolated preparation of these cellular inclusions with attendant catalytic activities intact, might justifiably afford a means of studying the metabolic efficiency of animals in various physiological states, as well as the *in vitro* consequences of inhibitor and activator compounds.

Toward this goal mitochondria were isolated from the livers of normal female rats, and were subjected to analysis in accord with the modified ferricyanide assay, as outlined in the materials and methods section. In addition, varying concentrations of sodium acetoacetate were included in the reaction mixture in order to determine their effects. A summarization of experiments conducted is included in Table 1.

TABLE 1

Effect of Varying Concentrations of Sodium Acetoacetate upon α-Ketoglutarate and Pyruvate Utilization by Normal Rat Liver Mitochondria

Treatment	μM Ferricyanide Reduced/mg mito. Protein/hr.		Average % Inhibition*
	Average*	Range	
α-Ketoglutarate	3.82	3.64-4.02	---
α-KGA + 2.5 mM AcAc	3.49	3.34-3.55	11.3
α-KGA + 7.5 mM AcAc	2.93	2.85-3.03	23.2
α-KGA + 12.5 mM AcAc	2.48	2.32-2.62	35.2
α-KGA + 25.0 mM AcAc	1.97	1.82-2.11	48.6
α-KGA + 50.0 mM AcAc	0.92	0.85-0.97	76.0
Pyruvate	1.10	0.97-1.19	---
Pyr. + 2.5 mM AcAc	1.03	0.91-1.13	6.1
Pyr. + 7.5 mM AcAc	0.88	0.78-0.95	19.3
Pyr. + 12.5 mM AcAc	0.79	0.69-0.84	28.2
Pyr. + 25.0 mM AcAc	0.65	0.59-0.72	40.5
Pyr. + 50.0 mM AcAc	0.31	0.27-0.37	69.9

*Average of duplicate observations of three replicated experiments.

α-KGA = α-Ketoglutarate
Pyr. = pyruvate
AcAc = acetoacetate

The standard enzyme assay system is defined as to contain:
ATP(Na) 6 μM
$MgSO_4$ 20 μM
EDTA(Na) 2 μM

Potassium Phosphate Buffer pH 7.4; 75 μM
Sucrose 250 μM

α-KGA or Pyr. 40 μM or 20 μM respectively
Defatted, washed, fluffy layer-free mitochondrial
Suspension 0.2 ml
Demineralized water to make 3.0 ml

The reaction was initiated by the timed addition of 0.7 ml of 0.00666 M potassium ferricyanide. The reaction was terminated by the timed addition of 1.0 ml of 10% TCA. The standard reaction time was 25 minutes, and 50 minutes respectively when α-KGA and Pyr. were the substrates. The standard reaction temperature was 25°C. Addition of acetoacetate was accomplished by deleting a portion of the water component.

In all tables
μM = μ moles
mM = millimoles

It should be observed that the activity of the α-ketoglutarate oxidation system is greater in comparison to that of the pyruvate oxidation sequence. The addition of acetoacetate at all concentrations tested, resulted in a decreased oxidation of ferricyanide. This is interpreted to mean that the α-keto acid substrate utilization was conjunctly inhibited. Support of this contention is furnished by the widely accepted opinion that ferricyanide can serve as an electron acceptor for reduced substrates. Increasing levels of inhibition occur at high, non-physiological concentrations of acetoacetate; however, it should be borne in mind that these liver mitochondria were isolated from normal non-ketotic animals. The total exposure time of these particles to acetoacetate varied from 27 to 52 minutes depending upon the substrate tested. It is conceivable that in ketosis, slow insidious changes, occurring over a period of time would more accurately reflect physiological alterations of the mitochondrial enzymatic activity.

In view of the effectiveness of cysteamine, the decarboxylated derivative of cysteine as a therapeutic agent in bovine ketosis (14, 15), the assessment of its effect on normal isolated rat liver mitochondria appeared desirable. The results of studies with variant levels of this compound are presented in Table 2. It is readily apparent that cysteamine exerted a stimulatory effect upon the reduction of ferricyanide monitored complete systems, and by

TABLE 2

Effect of Cysteamine Upon α-Ketoglutarate and Pyruvate Utilization by Normal Rat Liver Mitochondria

Treatment	μM Ferricyanide Reduced/mg mito. Protein/hr. Average*	Range	Average % Effect*
α-Ketoglutarate	3.97	3.56-4.34	----
α-KGA + 0.1 μM cystea.	4.59	4.12-4.99	+ 15.7
α-KGA + 0.2 μM cystea.	5.04	4.47-5.45	+ 26.8
α-KGA + 0.3 μM cystea.	5.76	5.04-6.42	+ 45.0
Pyruvate	1.05	0.93-1.08	----
Pyr + 0.1 μM cystea.	1.22	1.02-1.45	+ 16.8
Pyr + 0.2 μM cystea.	1.43	1.27-1.52	+ 36.4
Pyr + 0.3 μM cystea.	1.62	1.41-1.83	+ 55.1

*Average of duplicate observations of three replicated experiments.

Standard enzyme assay system as outlined in Table 1. Additions of cysteamine were made at the expense of the water component.

analogy the oxidation of both α-keto acid substrates was increased. Higher concentrations of cysteamine, not reported, were found to result in non-enzymatic reduction of the dye.

As cysteamine appeared to be a stimulant of α-keto acid oxidation by normal rat liver mitochondria, it became mandatory to study other sulfhydryl compounds in order to ascertain if this was a specific or general property. Experimental observations persuant to this matter are presented in Table 3. All sulfhydryl compounds tested with the exception of coenzyme A and mercaptopropionic acid apparently evoke a positive effect upon α-keto acid utilization. Cognition with regard to the significant stimulatory power of ergothioneine on pyruvate oxidation is evident (see Table 3).

If acetoacetate addition to normal rat liver mitochondrial preparations caused inhibition of α-keto acid substrate utilization as attested to by depressed ferricyanide reduction; and sulfhydryl compounds were effective in enhancing the utilization of these substrates; then, what effect if any, would there be on normal particles simultaneously exposed to these agents? The results of experiments with both inhibitor and activator substances present in the reaction medium are shown in Table 4 and Table 5. Significant reactivation of acetoacetate-inhibited normal liver mitochondrial α-keto acid oxidation was brought about by the concurrent addition of sulfhydryl compounds. At equimolar concentration, α-ketoglutarate oxidation was most effectively reactivated by cysteamine, while ergothioneine appeared most efficacious in the inhibited pyruvate system.

TABLE 3

Effect of Various Sulfhydryl Compounds upon α-Ketoglutarate and Pyruvate Utilization by Normal Rat Liver Mitochondria

Treatment	μM Ferricyanide Reduced/mg mito. Protein/hr. Average*	Range	Average % Effect*
α-Ketoglutarate	3.77	3.56-4.01	----
α-KGA + 0.3 μM Ergo.	4.90	4.64-5.18	+ 30.2
α-KGA + 0.3 μM GSH	4.79	4.47-5.14	+ 27.1
α-KGA + 0.3 μM ETOH-SH	4.55	4.29-4.88	+ 20.6
α-KGA + 0.3 μM CSH	5.57	5.27-6.02	+ 47.9
α-KGA + 0.3 μM Prop-SH	4.49	4.17-4.82	+ 19.1
α-KGA + 0.3 μM Homocyst.	3.85	3.63-4.09	+ 2.1
α-KGA + 0.3 μM CoASH	3.70	3.43-4.05	- 2.6
Pyruvate	1.05	0.96-1.13	----
Pyr. + 0.3 μM Ergo.	2.25	1.92-2.70	+102.6
Pyr. + 0.3 μM GSH	1.51	1.39-1.63	+ 43.2
Pyr. + 0.3 μM ETOH-SH	1.32	1.21-1.42	+ 25.2
Pyr. + 0.3 μM CSH	1.57	1.45-1.69	+ 49.2
Pyr. + 0.3 μM Prop-SH	0.99	0.91-1.06	- 5.6
Pyr. + 0.3 μM Homocyst.	1.26	1.15-1.36	+ 19.8
Pyr. + 0.3 μM CoASH	1.07	0.97-1.16	+ 0.8

*Average of duplicate observations of three replicated experiments.

α-KGA = α-Ketoglutarate
Pyr. = Pyruvate
GSH = Reduced glutathione
Ergo. = Ergothioneine
ETOH-SH = Mercaptoethanol
CSH = Cysteine
Prop-SH = Mercaptopropionic acid
Homocyst. = Homocysteine
CoASH = Coenzyme A

Standard enzyme assay system as outlined in Table 1. Addition of other components were made at the expense of the water increment.

TABLE 4

Effect of Sulfhydryl Compounds on Sodium Acetoacetate Induced Inhibition of α-Ketoglutarate Utilization by Normal Rat Liver Mitochondria

Treatment	μM Ferricyanide Reduced/mg mito. Protein/hr. Average*	Range	Average % Effect*
α-Ketoglutarate	3.94	3.87-4.02	----
α-KGA + 0.1 μM cystea.	4.47	4.40-4.58	+ 13.4
α-KGA + 0.2 μM cystea.	4.94	4.85-5.05	+ 25.3
α-KGA + 0.3 μM cystea.	5.61	5.49-5.76	+ 42.3
α-KGA + 0.2 μM Ergo.	4.87	4.78-5.03	+ 23.6
α-KGA + 0.23μM ± L(SH)$_2$	4.19	4.11-4.31	+ 6.7
α-KGA + 0.46μM ± L(SH)$_2$	4.35	4.26-4.48	+ 10.3
α-KGA + 50.0 mM AcAc	1.10	0.99-1.18	- 72.2
α-KGA + 50.0 mM AcAc + 0.1 μM cystea.	1.26	1.21-1.32	- 67.8
α-KGA + 50.0 mM AcAc + 0.2 μM cystea.	3.69	3.58-3.76	- 6.3
α-KGA + 50.0 mM AcAc + 0.3 μM cystea.	4.31	4.21-4.41	+ 9.2
α-KGA + 50.0 mM AcAc + 0.2 μM Ergo.	1.53	1.49-1.60	- 61.2
α-KGA + 50.0 mM AcAc + 0.23μM ± L(SH)$_2$	1.17	1.16-1.22	- 70.0
α-KGA + 50.0 mM AcAc + 0.46μM ± L(SH)$_2$	2.40	2.38-2.45	- 39.4

*Average of duplicate observations of three replicated experiments.

Cystea. = cysteamine
Ergo. = ergothioneine
±L(SH)$_2$ = D,L dihydrolipoic acid
AcAc = sodium acetoacetate
α-KGA = α-Ketoglutarate

Standard enzyme assay system as outlined in Table 1. Additions of other components were made at the expense of the water increment.

TABLE 5

Effect of Sulfhydryl Compounds on Sodium Acetoacetate Induced Inhibition of Pyruvate Utilization by Normal Rat Liver Mitochondria

Treatment	µM Ferricyanide Reduced/mg mito. Protein/hr. Average*	Range	Average % Effect*
Pyruvate	1.07	.95-1.14	----
Pyr. + 0.1 µM cystea.	1.22	1.09-1.29	+ 14.3
Pyr. + 0.2 µM cystea.	1.45	1.28-1.57	+ 36.3
Pyr. + 0.3 µM cystea.	1.63	1.43-1.73	+ 52.2
Pyr. + 0.2 µM Ergo.	2.07	1.81-2.20	+ 93.9
Pyr. + 0.23 µM ± $L(SH)_2$	1.25	1.11-1.33	+ 17.1
Pyr. + 0.46 µM ± $L(SH)_2$	1.61	1.43-1.70	+ 50.4
Pyr. + 50.0 mM AcAc	0.37	0.33-0.43	- 65.0
Pyr. + 50.0 mM AcAc + 0.1 µM cystea.	0.39	0.35-0.39	- 63.7
Pyr. + 50.0 mM AcAc + 0.2 µM cystea.	0.95	0.83-1.02	- 11.7
Pyr. + 50.0 mM AcAc + 0.3 µM cystea.	1.21	1.05-1.32	+ 13.6
Pyr. + 50.0 mM AcAc + 0.2 µM Ergo.	1.33	1.15-1.45	+ 24.6
Pyr. + 50.0 mM AcAc + 0.23 µM ± $L(SH)_2$	0.51	0.47-0.53	- 52.3
Pyr. + 50.0 mM AcAc + 0.46 µM ± $L(SH)_2$	0.58	0.49-0.63	- 46.0

Cystea. = cysteamine
Ergo. = ergothioneine
± $L(SH)_2$ = D,L dihydrolipoic acid
AcAc = sodium acetoacetate
Pyr. = pyruvate

*Average of duplicate observations of three replicated experiments.

Standard enzyme assay system as outlined in Table 1. Additions of other components were made at the expense of the water increment.

In order to determine if the activity of the enzyme systems responsible for the catalytic degradation of pyruvate and α-keto-glutarate were blocked in ketosis as proposed by Bach and Hibbitt (13), a study of these liver enzymes was initiated in the late-pregnant fasted guinea pig. Mature non-pregnant females similarly fasted served as controls. A summary of the results obtained appear in Table 6. It is easily observed that the enzymatic activity toward α-keto acid substrate utilization is conspicuously impaired in the ketotic state. Partial reactivation of _in vivo_ inhibited systems can be brought about _in vitro_ by the addition of sulfhydryl compounds to the reaction mixture. Results of these experiments can be found in Table 7. Dihydrolipoic acid or ergothioneine were especially beneficial on the pyruvate utilization system, while all three compounds tested on the α-ketoglutarate system were more equally effective. Thiamine pyrophosphate addition was inhibitory on all systems studied both in the ketotic and non-ketotic controls.

Enzymatic activity was also determined on the liver mitochondria obtained from a cow exhibiting low order spontaneous ketosis after parturition. In the course of the dry period the animal had been on an _ad libitum_ grain program. The blood ketone bodies and liver enzymic activities during the dry period (pre-ketosis), during ketosis and post-ketosis after treatment with cysteamine are

TABLE 6

The Effect of Fast Induced Ketosis in Pregnant Guinea Pigs on the Mitochondrial Utilization of α-Keto Acids in Comparison to Fasted Non-Pregnant Controls

Physiological State	Substrate	μM Ferricyanide Reduced/mg mito. Protein/hr. Average***	Range	Average % Effect***
N.K.*	α-KGA	1.16	1.01 - 1.32	——
K.**	α-KGA	0.62	0.57 - 0.68	- 46.6
N.K.*	Pyr.	0.74	0.61 - 0.84	——
K.**	Pyr.	0.40	0.32 - 0.51	- 45.9
N.K.*	Total Blood Ketones (mg %)	6.1	5.2 - 8.7	——
K.**	Total Blood Ketones (mg %)	24.0	19.8 -29.3	+393.4

N.K.* = Non-Ketotic (Non-pregnant fasted)
K.** = Ketotic (Pregnant fasted)
***Average of duplicate observations of six replicated experiments.

α-KGA = α-Ketoglutarate
Pyr. = Pyruvate

Standard enzyme assay system as outlined in Table 1. Temperature 30°C. Reaction time 50 minutes. The reaction was initiated by the addition of 0.7 ml of 0.00666M potassium ferricyanide.

TABLE 7

The Effect of Sulfhydryl Compounds, and Thiamine Pyrophosphate on the Mitochondrial Utilization of α-Keto Acids by Ketotic and Non-Ketotic Guinea Pigs

Treat-ment	Substrate	Sulfhydryl	μM Ferricyanide Reduced/mg mito. pro./hr. Aver-age*	Range	Average % Effect*
N.K.	α-Ketoglutarate		1.12	1.05 - 1.19	----
N.K.	α-KGA	+ 0.2μM cystea.	1.31	1.24 - 1.38	+ 17.2
N.K.	α-KGA	+ 0.2μM Ergo.	1.43	1.36 - 1.50	+ 27.8
N.K.	α-KGA	+ 0.46μM $\pm$ L(SH)$_2$	1.21	1.15 - 1.27	+ 8.3
N.K.	α-KGA	+ 0.2μM TPP	0.99	0.94 - 1.04	- 11.7
K.	α-Ketoglutarate		0.60	0.58 - 0.60	----
K.	α-KGA	+ 0.2μM cystea.	0.85	0.83 - 0.87	+ 41.4
K.	α-KGA	+ 0.2μM Ergo.	0.89	0.86 - 0.91	+ 47.4
K.	α-KGA	+ 0.46μM $\pm$ L(SH)$_2$	0.79	0.75 - 0.82	+ 31.1
K.	α-KGA	+ 0.2μM TPP	0.52	0.51 - 0.53	- 13.4
N.K.	Pyruvate		0.72	0.65 - 0.78	----
N.K.	Pyr.	+ 0.2μM cystea.	0.87	0.81 - 0.93	+ 21.2
N.K.	Pyr.	+ 0.2μM Ergo.	1.09	0.98 - 1.19	+ 51.4
N.K.	Pyr.	+ 0.46μM $\pm$ L(SH)$_2$	0.86	0.79 - 0.93	+ 20.3
N.K.	Pyr.	+ 0.2μM TPP	0.50	0.46 - 0.53	- 31.2
K.	Pyruvate		0.34	0.32 - 0.36	----
K.	Pyr.	+ 0.2μM cystea.	0.58	0.54 - 0.62	+ 70.9
K.	Pyr.	+ 0.2μM Ergo.	0.87	0.80 - 0.94	+156.0
K.	Pyr.	+ 0.46μM $\pm$ L(SH)$_2$	0.97	0.90 - 1.04	+185.1
K.	Pyr.	+ 0.2μM TPP	0.14	0.14 - 0.14	- 59.4

*Average of duplicate observations of two replicated experiments.
N.K. = Non-ketotic
K. = Ketotic
α-KGA = α-Ketoglutaric acid
Pyr. = Pyruvic acid
cystea. = Cysteamine
Ergo. = Ergothioneine
$\pm$ L(SH)$_2$ = D,L Dihydrolipoic acid
TPP = Thiamine pyrophosphate

Blood Ketones (mg %)	Experiment 1	Experiment 2
Pregnant Fasted Ketotic	25.7	29.3
Non-Pregnant Fasted Non-Ketotic	6.3	7.5

Standard enzyme assay system as outlined in Table 1.
Temperature 30°C. Reaction time 50 minutes. The reaction was initiated by the addition of 0.7 ml of 0.00666 M potassium ferricyanide. Additions of the other components were made at the expense of the water increment.

presented in Table 8. It is evident that the pre- and post-ketotic enzyme activity appears to be higher than when the ketone bodies were elevated. Another case of post partum ketosis which occurred following delivery of triplets is also recorded in Table 8. Similarly the enzymatic level for α-keto acid oxidation appears depressed in comparison to the non-ketotic condition brought about by cysteamine therapy. Further experimental evidence for the inhibition of pyruvic and α-ketoglutaratic oxidase activity in ketosis is provided in Table 9. Cows on a prepartum regimen of *ad libitum* grain feeding were subjected to a six-day fast two weeks post partum. Blood total ketone bodies and glucose levels are presented in Table 10. The data in Tables 8 and 9 demonstrate that a striking dimunition of enzymatic activity occurred in concert with depressed blood glucose and elevated blood ketone bodies.

Since there appears to be a relation between high ketone bodies and relatively low liver mitochondrial enzyme activity as determined in this investigation, it seemed obligatory to study these enzymes further with regard to possible activators and inhibitors. It appears that hormones have a profound effect on ketogenesis; this has been referred to in the literature review. What changes various hormones would have on isolated mitochondrial α-keto acid oxidase activity was now deemed essential. A number of hormones were

TABLE 8

The Effect of Spontaneous Ketosis and Cysteamine Therapy on the Level of α-Keto Acid Oxidase Activity of Bovine Liver Mitochondria

Condition or Treatment	In Vitro Substrate	μM Ferricyanide reduced/mg mito. prot./hr.	% Effect
Cow No. A			
Spontaneous Ketosis	α-KGA	.31	----
	Pyr.	.27	----
1 week after Cystea.	α-KGA	.39	+25.8
	Pyr.	.34	+25.9
Cow No. B			
1 month prior to parturition	α-KGA	.44	----
	Pyr.	.37	----
3 days post partum (ketosis)	α-KGA	.28	-36.4
	Pyr.	.19	-48.6
1 week after Cystea.	α-KGA	.40	- 9.1
	Pyr.	.35	- 5.4

Total blood ketone bodies	Mg. %
Cow No. A.	
Spontaneous ketosis	15.3
1 week after Cystea.	3.7
Cow No. B.	
1 month prior to parturition	6.2
3 days post partum	37.8
1 week after Cystea.	4.3

System:

Standard system as outlined in Table 1. A 0.4 ml. aliquot of the standard mitochondrial suspension rather than 0.2 ml. was used. Temperature 37° C. The reaction was initiated by the addition of 0.7 ml. of 0.00666 M potassium ferricyanide.

TABLE 9

Induction of Ketosis in Cattle by Fasting and the Effect on α-Keto Oxidase Activity of Liver Mitochondria

Animal	Treatment	μM Ferricyanide reduced/mg. mito. prot./hr. α-KGA	Pyr.	% Effect α-KGA	Pyr.
1	Prefast	.47	.46	——	——
	Nonfast, 6th day	.47	.41	0.0	- 10.9
2	Prefast	.43	.31	——	——
	Fast, 6th day	.15	.03	- 65.1	- 90.3
3	Prefast	.46	.38	——	——
	Nonfast, 6th day	.51	.46	+ 10.9	+ 21.1
4	Prefast	.44	.33	——	——
	Fast, 6th day	.34	.15	- 22.7	- 54.5

System:

Standard system as outlined in Table 1. A 0.4 ml aliquot of the mitochondrial suspension rather than 0.2 ml. was used. Temperature 37° C. Reaction initiated by the addition of 0.7 ml. of 0.00666 M potassium ferricyanide.

TABLE 10

Blood Glucose and Ketone Bodies during Induction of Ketosis in Cattle by Fasting

			Mg. % Ketone Bodies	Mg. % Glucose
Cow No. 1	day 0	(non-fast)	4.2	----
	day 3		2.9	62.5
	day 6		4.2	67.0
Cow No. 2	day 0	(fast)	7.7	----
	day 3		19.0	29.5
	day 6		41.8	10.6
Cow No. 3	day 0	(non-fast)	3.7	----
	day 3		2.6	75.1
	day 6		4.9	59.2
Cow No. 4	day 0	(fast)	6.2	----
	day 3		23.8	50.2
	day 6		40.2	21.2

effectual in causing changes in the enzymatic activity; the results of these investigations are summarized in Tables 11 and 12. It is evident that most hormones tested were inhibitory with the notable exception of insulin which was stimulatory in systems containing either α-keto acid substrate.

It is significant to note that cysteamine, the sulfhydryl compound effective as a therapeutic agent in bovine ketosis, is competent in reactivating *in vitro* hormonally inactivated normal rat liver mitochondrial α-ketoglutarate and pyruvate oxidase. These data are summarized in Tables 13-15.

With regard to mitochondrial swelling, it was observed that the addition of sodium acetoacetate resulted in enhanced protuberation of these particles when tested as outlined by Lehninger (323). The results of experiments on normal rat liver are given in Table 16.

TABLE 11

Effect of Hormones upon α-Ketoglutarate Utilization by Normal Rat Liver Mitochondria

Substrate	Hormone	μM Ferricyanide reduced/mg. mito.prot./hr. Average*	Range	Average % effect*
α-Ketoglutarate		4.04	3.95 - 4.12	------
α-KGA	Glucagon	3.25	3.23 - 3.27	-19.4
α-KGA	Insulin	4.78	4.64 - 4.93	+18.4
α-KGA	Diethylstilbestrol	1.49	1.41 - 1.60	-63.0
α-KGA	Progesterone	1.49	1.41 - 1.60	-63.0
α-KGA	Testosterone	3.61	3.51 - 3.73	-10.5
α-KGA	Hexesterol	1.09	1.02 - 1.19	-73.0
α-KGA	Cortisone Acetate	4.01	3.90 - 4.12	- .5
α-KGA	Corticosterone	3.99	3.88 - 4.06	- 1.1
α-KGA	Prednisolone	3.92	3.84 - 3.99	- 2.8
α-KGA	Estrone	4.34	4.29 - 4.40	+ 7.6
α-KGA	α-Estradiol	3.66	3.60 - 3.70	- 6.4
α-KGA	Androsterone	4.07	3.95 - 4.21	+ .7
α-KGA	Growth Hormone	2.48	2.47 - 2.50	-32.5
α-KGA	Hydrocortisone	4.14	4.08 - 4.19	+ 2.5
α-KGA	Thyroxine	1.62	1.53 - 1.74	-59.9
α-KGA	Triiodothyronine	1.96	1.88 - 2.01	-51.4
α-KGA	Dianabol	3.58	3.53 - 3.66	-11.4

* Average of duplicate observations of 3 replicated experiments.
Hormones concentrations, 5×10^{-5}M.

The standard system as outlined in Table 1 was used. Steroid hormones were added as propylene glycol soluble solutions replacing a portion of the water component of the standard system. Propylene glycol had no significant effect upon non-hormone-treated vessels. Pancreatic, pituitary and thyroid hormones were added as water soluble solutions replacing a portion of that constituent.

TABLE 12

Effect of Hormones upon Pyruvate Utilization by Normal Rat Liver Mitochondria

Substrate	Hormone	μM Ferricyanide reduced/mg. mito.protein/hr. Average*	Range	Average % Effect*
Pyruvate		0.93	0.89 - 0.98	——
Pyr.	Glucagon	0.35	0.32 - 0.39	-62.6
Pyr.	Insulin	1.25	1.19 - 1.33	+34.9
Pyr.	Diethylstilbestrol	0.26	0.23 - 0.29	-72.5
Pyr.	Progesterone	0.04	0.02 - 0.06	-95.7
Pyr.	Testosterone	0.37	0.34 - 0.36	-60.0
Pyr.	Hexesterol	0.04	0.02 - 0.05	-95.7
Pyr.	Cortisone Acetate	0.33	0.22 - 0.52	-76.3
Pyr.	Corticosterone	0.44	0.41 - 0.47	-53.1
Pyr.	Prednisolone	0.26	0.25 - 0.27	-72.1
Pyr.	Estrone	0.83	0.80 - 0.87	-11.1
Pyr.	α-Estradiol	0.50	0.48 - 0.53	-44.6
Pyr.	Androsterone	0.57	0.56 - 0.60	-38.2
Pyr.	Growth Hormone	0.63	0.59 - 0.66	-32.5
Pyr.	Hydrocortisone	0.57	0.53 - 0.61	-39.0
Pyr.	Thyroxine	0.02	0.00 - 0.03	-98.0
Pyr.	Triiodothyronine	0.03	0.02 - 0.04	-97.4
Pyr.	Dianabol	0.44	0.44 - 0.45	-52.3

* Average of duplicate observations of three replicated experiments. Hormone concentrations 5×10^{-5}M.

The standard system as outlined in Table 1 was used. Steroid hormones were added as propylene glycol soluble solutions replacing a portion of the water component of the standard system. Propylene glycol addition had no significant effect upon non-hormone-treated vessels. Pancreatic, pituitary and thyroid hormones were added as water-soluble solutions replacing a portion of that constituent.

TABLE 13

Effect of Cysteamine on Female Sex Hormone Induced Inhibition of α-Ketoglutarate Utilization of Normal Rat Liver Mitochondria

Treatment	μM Ferricyanide reduced/mg. mito.protein/hr. Average*	Range	Average % Effect
α-Ketoglutarate	4.03	3.97 - 4.12	----
α-KGA + .037 μM DES	3.05	2.97 - 3.15	-24.3
α-KGA + .111 μM DES	2.59	2.49 - 2.65	-35.7
α-KGA + .185 μM DES	1.34	1.18 - 1.51	-66.8
α-KGA + .037 μM DES +.1 μM Cystea.	3.31	3.24 - 3.35	-18.0
α-KGA + .037 μM DES + .2 μM Cystea.	4.26	4.14 - 4.40	+ 5.7
α-KGA + .037 μM DES + .3 μM Cystea.	4.46	4.39 - 4.52	+10.7
α-KGA + .037 μM Progest.	3.12	3.00 - 3.25	-22.8
α-KGA + .111 μM Progest.	2.55	2.47 - 2.60	-36.8
α-KGA + .185 μ mole Progest.	1.33	1.25 - 1.38	-67.1
α-KGA + .037 μ mole Progest. + .1 μ mole Cystea.	3.39	3.23 - 3.53	-16.0
α-KGA + .037 μ mole Progest. + .2 μ mole Cystea.	4.30	4.23 - 4.34	+ 6.6
α-KGA + .037 μ mole Progest. +.3 μ mole Cystea.	4.48	4.37 - 4.56	+11.0
α-KGA + .037 μ mole Hexest.	3.06	2.94 - 3.25	-24.2
α-KGA + .111 μ mole Hexest.	2.38	2.34 - 2.44	-41.1
α-KGA + .185 μ mole Hexest.	1.01	0.90 - 1.20	-74.9
α-KGA + .037 μ mole Hexest. + .1 μ mole Cystea.	3.15	3.05 - 3.27	-21.9
α-KGA + .037 μmole Hexest. + .2 μmole Cystea.	3.66	3.59 - 3.70	- 9.3
α-KGA + .037 μ mole Hexest. +.3 μ mole Cystea.	4.02	4.01 - 4.02	- .4

* Average of duplicate observations of three replicated experiments.

DES = Diethylstilbestrol
Progest. = Progesterone
Hexest. = Hexesterol
Cystea. = Cysteamine

The standard system as outlined in Table 1 was used. Hormones were added as propylene glycol soluble solutions replacing a portion of the water component of the standard system. Propylene glycol had no significantly demonstrable effect upon non-hormone-treated vessels.

TABLE 14

Effect of Cysteamine on Sex Hormone Induced Inhibition of Pyruvate Utilization of Normal Rat Liver Mitochondria

Treatment	μM Ferricyanide Reduced/mg. Mito.Protein/hr. Average*	Range	Average % Effect*
Pyruvate	0.97	0.93 - 1.02	----
Pyr. + .037 μM DES	0.71	0.69 - 0.73	-27.3
Pyr. + .111 μM DES	0.59	0.57 - 0.61	-39.4
Pyr. + .185 μM DES	0.21	0.20 - 0.23	-78.2
Pyr. + .037 μM DES + .1 μmole Cystea.	0.77	0.75 - 0.81	-20.3
Pyr. + .037 μM DES + .2 μmole Cystea.	0.93	0.90 - 0.96	- 4.9
Pyr. + .037 μM DES + .3 μmole Cystea.	1.01	0.97 - 1.07	+ 4.1
Pyr. + .037 μM Progest.	0.73	0.72 - 0.74	-24.9
Pyr. + .111 μM Progest.	0.50	0.47 - 0.54	-49.0
Pyr. + .185 μM Progest.	0.08	0.07 - 0.09	-92.0
Pyr. + .037 μM Progest. + .1 μmole Cystea.	0.82	0.79 - 0.86	-16.2
Pyr. + .037 μM Progest. + .2 μmole Cystea.	0.87	0.85 - 0.90	-10.3
Pyr. + .037 μM Progest. + .3 μmole Cystea.	0.94	0.90 - 0.99	- 3.7
Pyr. + .037 μM Hexest.	0.70	0.69 - 0.71	-28.3
Pyr. + .111 μM Hexest.	0.52	0.50 - 0.54	-46.9
Pyr. + .185 μM Hexest.	0.12	0.11 - 0.13	-87.6
Pyr. + .037 μM Hexest. + .1 μmole Cystea.	0.76	0.75 - 0.78	-21.3
Pyr. + .037 μM Hexest. + .2 μmole Cystea.	0.93	0.90 - 0.96	- 4.6
Pyr. + .037 μM Hexest. + .3 μmole Cystea.	1.03	1.00 - 1.07	+ 5.7

* Average of duplicate observations of 3 replicated experiments.

DES = diethylstibesterol
Progest. = progesterone
Hexest. = hexesterol
Cystea. = cysteamine

The standard system as outlined in Table 1 was used. Hormones were added as propylene glycol soluble solutions replacing a portion of the water component of the standard system. Propylene glycol had no significantly demonstratable effect upon non-hormone-treated vessels.

TABLE 15

Effect of Cysteamine on Thyroid Hormone Induced Inhibition of α-Ketoglutarate and Pyruvate Utilization by Normal Rat Liver Mitochondria

Treatment	μM Ferricyanide Reduced/mg.mito.protein/hr. Average*	Range	Average % Effect*
α-ketoglutarate	4.15	3.97 - 4.34	----
α-KGA + .037 μM Triiodoth.	3.63	3.53 - 3.78	-12.5
α-KGA + .111 μM Triiodoth.	2.78	2.71 - 2.91	-33.0
α-KGA + .037 μM Triiodoth. + .1 μM Cystea.	4.08	3.93 - 4.27	- 1.6
α-KGA + .037 μM Triiodoth. + .2 μM Cystea.	4.46	4.22 - 4.67	+ 7.5
α-KGA + .037 μM Triiodoth. + .3 μM Cystea.	5.11	4.89 - 5.30	+23.2
α-KGA + .037 μM Thyr.	3.18	3.01 - 3.36	-23.4
α-KGA + .111 μM Thyr.	2.53	2.39 - 2.72	-39.1
α-KGA + .037 μM Thyr. + .1 μM Cystea.	3.15	2.97 - 3.36	-24.0
α-KGA + .037 μM Thyr. + .2 μM Cystea.	3.50	3.38 - 3.66	-15.6
α-KGA + .037 μM Thyr. + .3 μM Cystea.	3.77	3.56 - 3.93	- 9.1
Pyruvate	1.02	0.97 - 1.08	----
Pyr. + .037 μM Triiodoth.	1.01	0.95 - 1.08	- 1.1
Pyr. + .111 μM Triiodoth.	0.72	0.69 - 0.76	-29.5
Pyr. + .111 μM Triiodoth. + .1 μM Cystea.	0.73	0.71 - 0.76	-28.8
Pyr. + .111 μM Triiodoth. + .2 μM Cystea.	0.75	0.73 - 0.79	-26.1
Pyr. + .111 μM Triiodoth. + .3 μM Cystea.	0.85	0.83 - 0.89	-16.3
Pyr. + .037 μM Thyr.	1.01	0.97 - 1.08	- .5
Pyr. + .111 μM Thyr.	0.72	0.69 - 0.76	-29.4
Pyr. + .111 μM Thyr. + .1 μM Cystea.	0.72	0.69 - 0.76	-28.8

(continued)

TABLE 15 (continued)

Treatment	µM Ferricyanide Reduced/mg.mito.protein/hr. Average*	Range	Average % Effect*
Pyr. + .111 µM Thyr. + .2 µM Cystea	0.87	0.83 - 0.94	-14.9
Pyr. + .111 µM Thyr. + .3 µM Cystea.	0.98	0.94 - 1.04	- 3.8

* Average of duplicate observations of three replicated experiments.

Triiodoth. = triiodothyronine
Thyr. = Thyroxine
Cystea. = Cysteamine

The standard system as outlined in Table 1 was used. Hormones were added as water-soluble solutions replacing a portion of the water component of the standard system.

TABLE 16

The Effect of Acetoacetate Addition on the Spontaneous Swelling of Normal Isolated Rat Liver Mitochondria

Time (min.)	Treatment	$-\Delta D_{520} \times 10^3$ *
	Normal (Spontaneous)	
12		24.9 - 29.2 **
24		77.5 - 79.3
36		96.9 - 101.7
	Normal + 50 mM AcAc	
12		66.9 - 70.4
24		98.5 - 103.7
36		112.7 - 115.8

* - $D_{520} \times 10^3$ = change in optical density at 520 mμ from initial multiplied by 1000.

** Range of three experiments

IV. DISCUSSION OF RESULTS

The effect of sodium acetoacetate, sulfhydryl compounds and hormones on isolated normal rat liver mitochondria have been studied. Liver mitochondrial α-keto acid oxidase activity has been measured in order to determine if this activity was variable during pathological conditions.

The data concerning the effect of sodium acetoacetate on normal rat liver mitochondria are in accord with existing evidence that this compound is a toxic metabolite (see Table 1). The consequences of sodium acetoacetate administration have been elaborated upon in section E of the literature review. Inhibitory results herein obtained on α-keto acid oxidation helps to explain the observation of Nath and Chakrabarti (161), that pyruvate levels in the blood and urine are increased after sodium acetoacetate injection in the intact animal. The increased urinary pyruvate may be a reflection of excessive blood titer and subsequent transcendence of the renal threshold. Bach and Hibbitt (13) first noted that the pyruvate and α-ketoglutarate blood levels were increased in bovine ketosis and proposed a block in the Krebs cycle to explain their findings. The results of this study on both the ketotic guinea pigs, (Table 6) and the ketotic cattle (Table 8-10) indeed demonstrate that the mitochondria which contain the enzymes responsible for α-keto

acid substrate utilization have depressed activity during that pathological state. It should be emphasized that acetoacetate and acetate have the capacity to form thioesters with many mono- and dithiol-compounds such as reduced glutathione, pantetheine, reduced coenzyme A, monothioloctanoic acid, 6,8 dithioloctanoic (dihydrolipoic) acid, and dimercaptopropanol, (BAL) (316, 324-330). Reduced glutathione levels of blood have also been noted to be depressed subsequent to acetoacetate administration (163-165).

Cysteamine, a sulfhydryl compound, has been found to cause enhanced mitochondrial enzymatic utilization of both α-keto acid substrates (Table 2). This novel effect is shared by other sulfhydryl compounds such as reduced glutathione, ergothioneine, cysteine, homocysteine and mercaptoethanol (Table 3). The distinct activation caused by ergothioneine on pyruvate oxidation is to be particularly noted in view of the unknown metabolic niche for this compound. It is proposed that ergothioneine specifically may act as a co-factor in pyruvate utilization by the pyruvic acid oxidase-enzyme complex. The possibility that ergothioneine causes stimulation of the oxidation of DPNH by peroxidase as reported by Klebanoff (331) may tend to stimulate the whole complex toward greater activity by increasing DPN concentration. The non-effectiveness of coenzyme A to enhance normal mitochondrial activity may be a reflection of an inability to pass through the mitochondrial membrane barrier.

The salutary effects on acetoacetate inhibited isolated normal rat liver mitochondria and the reactivating ability of sulfhydryl compounds on isolated ketotic guinea pig mitochondria have been recognized (see Tables 4 and 7). This lends further credence to the possibility that the presence of excessive acetoacetate is intimately related to the availability of essential sulfhydryl compounds and/or groups.

Bach and Hibbitt (14, 15) have theorized that cysteamine was an effective therapeutic agent by virtue of its being purportedly able to act in lieu of coenzyme A, which had been previously reported by Gallagher (45) to be at sub-normal levels in ewes suffering from ketosis. This hypothesis appears to be rather untenable in view of the recent report by Wieland and Weiss (332), who have observed increased levels of acetyl-CoA in the livers of ketotic alloxan diabetic rats. Further evidence counter to the view of CoA lack is supplied from the recent work of Carter and Hockaday (194) who noted that pantothenic acid-deficient rat liver slices had lower oxygen uptake and also depressed ketogenesis.

It is proposed that acetoacetate is inhibitory on the pyruvate and α-ketoglutarate-oxidizing systems of the mitochondria *in vitro* and *in vivo* (in ketosis) by virtue of its ability to compete for indispensable sulfhydryl groups. This contention is supported by the observations that these enzymes contain essential sulfhydryl groups (240-245, 293), that they require sulfur-containing co-factors

for their activity (212-214) and that mitochondrial permeability is intimately controlled by the presence of sulfhydryl acceptor sites on the membrane surface (311). A number of possibilities concerning the mode of acetoacetate inhibition are postulated, all involving sulfhydryl groups: (a) that a necessary group on the enzyme itself is attacked by the inhibitor; (b) that a sulfur-containing co-factor such as thiamine pyrophosphate, α-lipoic acid, or CoA, is preferentially bound by the ketone body; (c) that the mitochondrial membrane is in some way altered, resulting in lack of permeability to substrate; or (d) that the mitochondrial membrane is damaged, resulting in swelling and disorganization of the vital spatial arrangement within the particle. Acetoacetate *in vitro* has been shown in this investigation to cause swelling of isolated normal rat liver mitochondria (see Table 16). However, all possibilities remain valid since the normally-occurring sulfhydryl compounds, dihydrolipoic acid and ergothioneine have been demonstrated to be effective in promoting activity of *in vivo* and *in vitro* depressed systems. Additional studies need be undertaken to fully explain the site of inhibition of aceto - acetate on α-keto acid oxidation.

Hormones have been shown to be potent metabolic control agents *in vivo* and *in vitro.* Their therapeutic action in bovine ketosis has been expounded in Section B of the literature review, while the

popularly-held concept of pituitary-adrenal insufficiency and other hormonal imbalances as related to ketosis has been reviewed in Section A. Some hormones have been classified as ketogenic, whereas other appear to exert an opposite effect. Conditions resulting in increased and depressed ketogenesis have been covered in Sections D and F, respectively. Hormonal alteration of α-keto acid metabolism has been alluded in Section H, and hormone-induced mitochondrial swelling as a plausible explanation of the cogent changes effected by these compounds was reviewed in Section I.

Investigations were conducted on normal isolated rat liver mitochondria and their ability to utilize pyruvate and α-ketoglutarate in the presence of various hormones (Tables 11-15). Of all the hormones tested, only insulin exerted a stimulatory effect on α-keto acid oxidation when added to the _in vitro_ reaction mixture. Insulin has been noted by other investigators to elicit an antiketogenic effect (89, 179, 180), and to be effective in causing a decrease in the hyperpyruvecemia associated with insulin lack (251-254, 266). Glucagon appeared to have an inhibitory effect which was particularly marked upon the pyruvate oxidation capacity. This appears to be in harmony with the report of Butturini and co-workers (267), who noted that this hormone caused hyperpyruvecemia in humans after its administration. Whether the insulin and glucagon effects noted

are due to enzymatic stimulation and inhibition or represent altered mitochondrial permeability, remains to be elucidated.

The female sex hormone progesterone, and the synthetic estrogens, hexesterol and diethylstilbesterol were found in this investigation to exert a powerful inhibitory effect upon the mitochondrial oxidation of both α-keto acid substrates under investigation. The glucocorticoid class of steroids such as cortisone, hydrocortisone, corticosterone, and prednisolone were found to have only minimal effect on α-ketoglutarate activity (see Table 11), but all had a potent inhibitory effect on mitochondrial pyruvate utilization. This enigmatic observation may be related to the heretofore unreported effectiveness of ergothioneine. It induced an increase in pyruvate oxidation, which may be a reflection on DPN concentration within the mitochondrion. Various female sex and glucocortical steroids have been shown to be potent inhibitors of DPN-linked dehydrogenases (333-344). Whether this type of inhibition is operative in α-keto acid utilization remains to be elucidated in view of the effectiveness of sulfhydryl compounds in causing remission of _in vitro_ inhibition (see Tables 11 and 14).

Other hormones eliciting powerful inhibitory action on the normal mitochondrial α-keto oxidase systems studied were the thyroid hormones, thyroxine and triiodothyronine (Table 12). Their effect could be reversed by the _in vitro_ simultaneous inclusion of cysteamine

in the reaction mixture (Table 15). It has been previously reported that these thyroid hormones are inhibitors of isolated dehydrogenases, especially the malic, glutamic, alcohol, lactic, and glyceraldehyde 3-phosphate dehydrogenases (345). Emmelot and Bos (346) have reported that thyroxine inhibited mitochondrial glutamic dehydrogenase by causing DPN release from the enzymatically active sites. It is conceivable that addition of sulfhydryl groups to inactivated systems can cause reactivation as evidenced by the results of this investigation, which are presented in Table 15. The mechanism of this might be the reversal of binding of the thyroxine to the protein at sulfhydryl sites. It has already been noted that thyroxine binds strongly with mitochondria and microsomes (313, 314), and that this hormone is a strong swelling agent for isolated mitochondria (290).

The incomplete and possibly erroneous hormonal picture as reported for bovine ketosis has already been referred to. In short, there appears to be low circulating insulin in bovine ketosis (39, 40) as well as depressed protein-bound iodine concentration (27), in conjunction with increased circulating 17-hydroxycortocoid titer (27, 28). Bovine ketosis has been classified as an adreno-cortical insufficiency brought about by the stress of parturition (16-19).

It appears to be in the interest of clarity to discuss the term adrenal cortical insufficiency as applied to bovine ketosis. It has been reported in animals that the pyruvate level is low in

adrenal insufficiency (347). Frawley (348) has failed to note any consistent alteration of pyruvate metabolism in adrenal cortical insufficiency in man. In regard to the depressed utilization of pyruvate and α-ketoglutarate utilization as shown for liver mitochondria isolated from ketotic subjects in this study, and the hyperpyruvecemia and hyperketoglutaremia noted by Back and Hibbitt (13) in bovine ketosis, the term "adrenal cortical insufficiency" (16-19) as used to describe the condition existing in bovine ketosis appears to be a misnomer, and should be dropped from useage. The endocrine disorder that can result in hypoglycemia and which parallels the adrenal changes observed in bovine ketosis has been indicated as congenital adrenal hyperplasia (349). The fundamental disturbance in this malady seems to be an inability of the adrenal cortex to produce normal amounts of hydrocortisone. This serves as a stimulus for increased production of ACTH. The adrenals become hyperplastic and produce a large quantity of other steroidal material, which appears in the urine as 17-ketosteroids, (350).

Awareness as to the true physiologic status of the circulating hormones is just becoming evident. It would seem that thyroxine binds to the serum proteins (351-356) which serve to transport this hormone from the site of elaboration to the target tissue (357). It has been stated by Deiss (357) that the level of "free" non-protein bound

thyroxine is quite small, but has great physiologic importance, since it is this fraction that is considered to be in contact with hormone responsive sites. This, then, causes one to reflect upon the true worth of serum protein-bound iodine determinations, as well as other serum hormonal levels, which may obviously not allow one to deduce the true concentration of hormone at the locus of action. This proposition is further supported by the report of Booth et al. (358) who states that the physiological activity of cortisol is thought to be determined by the free, non-protein bound hormone.

It will suffice to state that meaningful hormonal information persuant to the ketosis problem await the more refined measurement of free non-protein bound hormone titers at the enzyme sites.

V. CONCLUSIONS

It may be concluded that:

1. The enzymatic activities relative to the utilization of α-ketoglutarate and pyruvate in isolated normal rat liver mitochondria can be inhibited by the *in vitro* inclusion of sodium acetoacetate to the reaction mixture.

2. The liver mitochondria of ketotic pregnant fasted guinea pigs and fast-induced ketotic liver particles of cows, exhibit diminished activity toward both α-ketoglutarate and pyruvate substrates.

3. Acetoacetate appears to act as a swelling agent on isolated normal rat liver mitochondria.

4. Sulfhydryl compounds generally exert a stimulatory effect upon mitochondrial α-keto acid oxidase activity. Cysteamine and ergothioneine appear particularly effective.

5. Ergothioneine may participate in α-keto acid oxidation in the role of a cofactor.

6. Hormones have a marked effect upon mitochondrial enzymatic activity. Insulin appears to be stimulatory, while glucagon, female sex hormones, glucocorticoids and thyroid hormones all are of an inhibitory nature. Hormonal effects appear to vary depending on the α-keto acid substrate used.

7. Sulfhydryl compounds are effective in reversing the *in vivo* and *in vitro* effects of ketosis and acetoacetate on isolated liver α-keto acid oxidation.

8. Sulfhydryl compounds have been noted to be efficacious in reactivating hormonal caused blockage of the mitochondrial enzymatic activities studied.

VI. SUMMARY

In brief, ketosis in the bovine has been noted to occur in conjunction with elevated blood pyruvate and α-ketoglutarate. It appears from these studies that this observation is the result of depressed enzymatic activity of the liver mitochondria, in particular, the reactions involved in the utilization of these intermediates. Cysteamine has been reported to be an effective therapeutic agent in bovine ketosis. Experimental evidence is presented herein in support of the newly-derived proposition that sulfhydryl compounds including cysteamine can stimulate normal as well as pathologically deranged mitochondrial pyruvate and α-ketoglutarate oxidation. Ergothioneine has been noted in these investigations to exert a potent stimulatory effect upon pyruvate oxidation by normal rat liver mitochondria. A hypothesis has been advanced in that this compound of unknown biochemical role may be acting in the capacity of a co-factor. Hormones have been noted in these studies to exert profound effects upon normal isolated rat liver mitochondria. Insulin can cause enhanced mitochondrial keto acid utilization. Thyroid hormones, female sex hormones and certain steroids are reported to be inhibitory. It is conceivable that these hormonal changes represent an effect either upon mitochondrial membrane or upon internal mitochondrial enzymes. In either event the enzymatic regulation by hormones _in vitro_ has been strikingly portrayed. Sulfhydryl reactivation of hormonally

blocked mitochondrial enzyme systems has been achieved. It is proposed that the hormonal control of enzymatic processes may be related to sulfhydryl groups. The need for continued investigation in ketosis at molecular level has been emphasized.

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ABSTRACT

The mitochondrial utilization of α-ketoglutaric and pyruvic acids have been found to be depressed in guinea pig and bovine ketosis. Addition of sodium acetoacetate to the reaction medium resulted in depressed oxidation of pyruvate and α-ketoglutarate of isolated normal rat liver mitochondria.

Sulfhydryl compounds, especially cysteamine and ergothioneine have proven stimulatory upon the α-keto acid oxidation of normal rat liver mitochondria. Ergothioneine addition has been noted to cause maximal stimulation of pyruvate utilization. The possible significance of this observation is discussed.

Sulfhydryl compounds were effective in stimulating the depressed oxidation of α-keto acid substrates in pathological conditions. Moreover, this class of compounds also caused reactivation of sodium acetoacetate inhibited normal systems. The apparent interrelationship between sulfhydryl groups and ketone bodies, especially acetoacetate was noted. Acetoacetate may be inhibitory on α-keto acid oxidation by affecting essential sulfhydryl groups either on the mitochondrial membrane surface, or by acting on the intra mitochondrial enzyme complex.

Hormonal effects upon the enzymatic activity of isolated normal mitochondria were also studied. Thyroid hormones, female sex hormones, and certain adrenal steroids caused sulfhydryl reversible inhibition of mitochondrial α-keto acid oxidation. Insulin addition caused *in vitro* stimulation with both substrates. The possible effect of these biochemical materials upon mitochondrion integrity were investigated.