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Relationship of ketone bodies to blood lipids in the rabbit

H Martin Blacker
University of Nebraska Medical Center

THE RELATIONSHIP OF KETONE BODIES TO BLOOD LIPIDS IN THE RABBIT

H. Martin Blacker

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College of Medicine, The University of Nebraska

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Introduction

The infant looks at his father...he observes the fact that this wonderful figure exists...he accepts that fact completely...without analysis...without question...without reservation.

The child becomes an adolescent...he begins to question his father...he begins to mentally dissect... ...to criticize...to break down into little pieces to see the stuff of which fathers are made.

The adolescent grows up into a mature adult... ...slowly, painfully the pieces are put back together and once more he looks at his father...this time with understanding.

In a sense the scientific method is like this cycle of growth. We observe a phenomenon...we begin to dissect it...to break it down into its elemental units. Then slowly we begin the process of synthesis... ...of putting the fragments of our knowledge of the pieces together again. With this synthesis comes our understanding.

A corollary to the above is that the more complex the phenomenon the longer and more extensive must be the period of "dissection".

With respect to our knowledge of the human organism

we are still in our scientific adolescence. The obvious reason is the endlessly complex nature of the organism. Each grain of truth uncovered by careful investigation contributes only a minute measure to the whole of scientific truth about this organism.

One of the paths of investigation involves the elucidation of the mysteries of metabolism. One facet of metabolism is the way in which the organism compensates for a disturbance in its ability to garner energy. When one pathway is blocked, the organism seeks another to supply its needs.

This investigation was undertaken to attempt to throw light on one of the ways in which the organism invokes other pathways of metabolism in disease states.

The work herein is an attempt to contribute something to that process of dissection referred to above. It will remain for future investigation to effect a synthesis so that we may look upon this organism with understanding. Happily, the very process of dissection is exciting.

Literature Review

The Discovery of Ketone Bodies

In 1857, Petters (1) examined the urine of diabetics and recognized the presence of acetone. He looked upon acetone as an intermediate in the metabolism of glucose.

Gerhardt described the presence of acetoacetic acid in diabetic urine in 1865 (3).

The presence of an asymmetric carbon atom in the third ketone body, beta-hydroxybutyric acid, led Kulz to its discovery in 1884 (4).

The above represent just three of the many investigations which elucidated the presence of three ketone bodies in the urine of diabetics between the years 1857 and 1884 (5).

Site of Ketone Body Production

Numerous investigations have lead to the conclusion that the liver is the organ chiefly responsible for ketone body production. In 1926, (6) and in 1928,(7) Snapper, and Grunbaum perfused livers, skeletal muscles, lungs, and kidneys and found that only the liver forms appreciable quantities of ketone bodies.

In 1931 Himwich and others (8) investigated the function of the liver, striated muscle, and the gastro-

intestinal tract by means of analyses of afferent and efferent blood samples for total acetone substances. They found that the liver was the most constant source of acetone substances regardless of the action of the other organs. They drew blood samples simultaneously from striated muscle and the gastro-intestinal tract and showed that these organs decreased the blood concentration of total acetone substances in most experiments. In 1931, these same authors (9) extended their observations to include the heart, brain, and testicle of the dog. They found that the heart removed ketone bodies from the blood in 8 of the 15 experiments, and that in 3 of the 15 experiments the heart added ketone bodies to the blood. The brain, on the other hand, made no change in the ketone acids in 18 of 23 profusion experiments. The testicle removed acetone from the blood in 6 experiments and made no change in 8 of 15 experiments.

Jowett and Quastel (10) in 1935 found that kidney, spleen and tests produce acetoacetic acid in small quantities from fatty acids, but that brain gave no measurable production. All of these tissues, according to these authors, produce acetoacetic acid from d,l - beta-hydroxybutyric acid.

In 1928, Chaikoff and Soskin (11) showed that there was a rapid decline of blood ketones following removal of the liver.

Although I intend to review the hormonal influences on ketosis later in this paper, it is interesting to note that in connection with hepatic production of ketones Mirsky (12) found that the ketogenic principle of the anterior lobe of the hypophysis acts solely through the liver and not on extra-hepatic tissues. When the liver was removed, there was no ketosis; when it was present, there was a marked ketosis. He further concluded that the muscles did not contribute in any measurable degree to the accumulations of ketones in the blood.

It was found by the investigations of Magnus-Levy 1908, (14) and Lusk in 1928 (15) that the ketone bodies must originate in the fatty acids of fat and possibly to a limited extent from part of some amino acids in protein.

It should be understood, however, that although the liver is the only organ capable of adding significantly to blood levels of ketones as shown by the above investigations, the ketones in general and acetoacetate in particular are formed by other organs,

e.g. in kidney, (8, 9, & 10).

Further amplifying this last point, i.e. acetoacetate production in the kidney, Medes, Floyd and Weinhouse (16), studied the metabolism of acetate by isotopic dilution methods. They found that of the acetate that disappeared, 44 per cent was incorporated into acetoacetate. However, the oxidation of acetoacetate by the renal tissue was so rapid that the accumulation of this ketone in kidney tissue was evanescent.

Interrelationship of Ketone Bodies

The fact that the ketone bodies are interconvertible has been shown by numerous investigations performed at the beginning of this century.

In 1910, Blum (17), reported the excretion of l, beta-hydroxybutyric acid in the urine following large subcutaneous injections of the sodium salt of d,l - beta-hydroxybutyric acid.

Four years later, Marriott (18) demonstrated an increase of blood beta-hydroxybutyric acid concentration coincident with its excretion in the urine following intravenous administrations of sodium acetoacetate. He also demonstrated a rise in beta-hydroxybutyric acid in tissues in the same experiment.

Marriott's experiments offered only an inference of the acetoacetic acid-beta-hydroxybutyric acid conversion. He did not show the relationship on a sound quantitative basis. It remained for Wilder (19) to demonstrate this. Using sodium beta-hydroxybutyate obtained from diabetic urine, Wilder set up a constant infusion and measured the appearance of the beta-hydroxybutyrate in urine. According to Wilder's calculations, there was no direct evidence to indicate that any of the injected beta-hydroxybutyrate was oxidized to acetoacetate. On the other hand the continuous infusion of acetoacetate resulted in an almost quantitative conversion of this substance into beta-hydroxybutyrate, the latter having been measured in urine.

When Chaikoff and Soskin (11) administered acetoacetate to eviscerated dogs, they noted a rapid accumulation of beta-hydroxybutyrate with its subsequent disappearance.

According to Snapper and Grumbaum (20), the perfusion of acetoacetate through liver results in its conversion in part to beta-hydroxybutyrate without appreciable utilization.

Thus there is a considerable body of evidence --

more than that cited in this paper -- that the conditions in tissues favor the reduction of acetoacetate to beta-hydroxybutyrate (5).

What of the conversion of acetoacetate to acetone? Prior to the work of Plaut and Lardy (22) it was generally conceded that the decarboxylation of acetoacetic acid to acetone was irreversible. These investigators showed by means of isotopic labeled acetone that the conversion of the latter to acetoacetate was to a limited extent reversible. The reaction involved CO_2 fixation. Deuel, (2) reports, however, that this pathway is a minor one.

Blood Levels of Ketones

According to Barnes and Wick (23), the concentration of ketone bodies in the blood of normal individuals on a general diet is practically insignificant.

The ketones appear in largest quantities in cases of severe diabetes and with normal subjects only when fasting or when the food carbohydrate is greatly restricted according to MacKay, (13).

That ketone levels in blood can reach extremely high concentrations was demonstrated by Allen, Stillman, and Fitz in 1919 (24) who measured ketones (calculated as acetone) of 368 milligrams per cent in the blood of

an individual in diabetic acidosis.

Earlier in this review the fact was brought out that conditions in tissues favor the reduction of acetoacetate to beta-hydroxybutyrate. Evidence of this favoritism towards beta-hydroxybutyrate might be reflected by the fact that according to Mirsky and Broh-Kahn (25) 75 per cent of the ketone bodies of the blood are present in the form of beta-hydroxybutyrate.

With regard to ketone homeostasis, Engel (26) concludes that blood levels of ketone bodies are largely determined by their rate of production and release by the liver because the rate of ketolysis by peripheral tissues does not fluctuate within as wide limits as does the rate of production. Renal excretion varies under different physiologic and pathologic conditions and thus ketonuria may not always be an adequate reflection of ketonemia.

Ketolysis

Using the tissue-slice technique, Quastel and Wheatley (27) demonstrated that acetoacetic acid was not further oxidized by the liver. This same observation was made by Snapper and Grunbaum (20), using perfusion techniques with liver.

In contrast to liver, the extrahepatic tissues

utilize the ketone bodies with ease. This had been demonstrated by numerous investigations. Snapper and Grunbaum (21) perfused kidney and muscle with beta-hydroxybutyrate and found that these organs were able to utilize this substance quite readily. They were unable to demonstrate this in liver.

Marriot's work, (18) besides providing evidence for the interconversion of ketones, showed the ability of the tissues to utilize ketones.

Barnes and co-workers (28) measured the ketone body utilization in the normal and diabetic goat, dog and rabbit. Their method was that of simultaneous ketone body and oxygen arterio-venous differences. The average fraction of the oxygen difference utilized by burning ketones was 44 per cent. They found that normal animals injected with beta-hydroxybutyric acid showed no greater utilization of ketones than animals with diabetic ketosis. This latter finding implies that ketone utilization is independent of the presence of carbohydrate.

There is evidence that the higher the concentration of ketones in blood the higher the rate of utilization by peripheral tissues. Chaikoff and Soskin (11) using depancreatized dogs injected acetoacetic acid into these animals after also removing the liver and kidneys and

followed the blood level of ketones. Following the injection the blood ketones rose to over 100 mg. per cent from which there was a rapid fall within two or three hours to 10-30 mg. per cent. Following this initial rapid fall there was a tendency to level off. These results suggest that utilization rate at high levels of ketosis is very rapid but is not of very great magnitude at low levels of ketonemia.

Further evidence that ketone utilization in peripheral tissues is independent of the presence of carbohydrate is found in the work of Blexenkrone-Moller (29). This author found no significant difference in the utilization of ketone bodies in perfused hind quarters of normal and diabetic cats.

Barnes and Drury in 1937 (30) and Waters, Fletcher, and Mirsky in 1938 (31) found that carbohydrate does not influence the utilization of ketone bodies by the heart lung preparation.

That the utilization of ketone bodies may be the prime mechanism for meeting energy requirements in some instances was found by Barnes and co-workers (28) and Crandall, Ivy, and Ehni (32). These investigators found that in certain ketogenic states from 30-80 per cent of the energy requirements of the tissues may be

supplied by the combustion of the ketone bodies. Wick and Drury in 1941 (33) found that under certain experimental conditions as much as 90 per cent of the calories consumed by the organism may be accounted for by the ketone bodies.

Ketones and Hormones

Several groups of investigators almost simultaneously discovered and studied the ketogenic activity of anterior pituitary extracts including Burn and Ling (34), Black, Collip and Thomson (35) and Houssay and Rietti (36).

The relationship of the adrenal medulla and ketone metabolism has been studied by Wool and co-workers (37) who found that the medullary portion of the gland was not essential for ketosis. Their evidence for this observation came from studies in the rat in which ketonemic response to insulin hypoglycemia occured despite an adrenomedullectomy.

The administration of epinephine had a depressing effect on the ketonemia of the rat according to Engel (26). In humans, however, Warming-Larsen (38) described a ketonemic response or a lack of any effect of epinephrine.

The relationship between the adrenal cortex and

ketosis was rather obscure due to the conflicting reports in the early literature. Engel (26) attributes this to the fact that ketonuria was used as the basis for the estimation of ketosis and the influence of abnormal renal function in the presence of adrenal insufficiency was not taken into account. Adrenalectomy was observed to depress ketonuria but the simultaneous measurement of blood ketones showed that the latter was not depressed as much as anticipated from the urine ketone values according to Nelson (39) and MacKay et. al. (40). The studies of Engel and Hewson (41) indicate that a failing peripheral circulation by itself inhibits ketonemia and it is this latter effect that obtains from adrenalectomy that is believed to have introduced distortion into the estimate of ketonemic action of the adrenal steroids themselves. Engel and Engel (42) showed that adrenalectomized rats maintained with saline and DOCA during fasting exhibit a slight but significant increase of ketonemia over their normal controls. Treatment with cortisone reversed this elevation of ketones over normal. Thus, Engel concludes, the adrenal cortex, like the adrenal medulla is not essential for the ketosis of fasting or hypoglycemia. In fact cortisone and a slow infusion of ACTH as shown by

Kinsell, et. al. (43) actually inhibits a fasting ketosis.

Scott and Engel (44) found that cortisone and hydrocortisone suppress the ketosis of cold stress. However, these steroids do not depress the ketonemia secondary to insulin-induced hypoglycemia in rat (26) or in man (45). Thus, concludes Engel (26) the adrenal cortex is not only not essential for ketosis, but the glusocorticoids, under certain circumstances actually inhibit ketosis.

Engel (26) admits that the actual mechanism by which the adrenal cortex mediates its effects on ketone metabolism is difficult to delineate. One of the largest areas of disagreement is in regard to the corticoid's influence on fat anabolism and catabolism. That adrenal hormones inhibit lipogenesis or more correctly that the lack of corticoids favors lipogenesis is reflected in the work of Welt and Wilhelmi (46). These investigators found that adrenalectomized rats had a higher rate of lipogenesis on a high carbohydrate diet than the controls. These observations are supported by the work of Gurin and Brady (47) who found that adrenalectomy and hypophysectomy restored lipogenesis in diabetic animals.

The exact opposite view i.e. that corticoids are necessary for lipogenesis is taken by Lipsett and Moore (48) and Perry and Bowen (49, 50) who found impaired lipogenesis from acetate in the livers of adrenalectomized rats.

Engel (26) offers a reasonable explanation for the mild ketosis of fasting in the animal whose adrenals have been removed, and the suppression of ketosis following administration of glucocorticoids. Initially, in the adrenalectomized animal, fasting results in a rapid depletion of liver glycogen. This is followed by an acceleration in the catabolism of fat. The latter along with the depletion of liver glycogen results in an increased amount of acetyl CoA available for pathways of ketogenesis. Normally the catabolism of protein as the fast continues, helpfully mediated by the corticoids, provides more amino acid fragments for gluconeogenesis. The latter also provide more Krebs cycle intermediates, a situation that facilitates accommodation of Acetyl CoA into final oxidative pathways. In the absence of the corticoids, there would be less gluconeogenesis and a diminished availability of amino acids for conversion to Krebs cycle intermediates, and hence, a less efficient Krebs cycle. The latter situation results

in a diminished accommodation of acetyl CoA into final oxidative pathways, directing it into pathways of ketogenesis (26).

The role of the thyroid in ketone metabolism is not entirely clear. However, a few studies have been done. Mirsky and Broh-Kahn in 1936 and 1937 (51, 52) found that an increase in the metabolic rate induced by hyperthyroidism increases the rate of utilization of glucose. Consequently injected beta-hydroxybutyrate is utilized at an increased rate under these conditions.

The hypophysis has several diverse effects, several of which relate to ketone metabolism. Many of these effects have become associated with a fraction of the anterior pituitary possessing growth activity. In fact most investigators believe that these effects are specific properties of "growth hormone" (26). However, Astwood (53) and Engel (54) found that the metabolic activities attributed to growth hormone, including mobilization of fat to liver, hyperlipemia, depression of the R.Q., hypoglycemia in the fasted animal, and antiinsulin activity, may be as much or more a property of a protein or polypeptide that is chemically more like ACTH than somatotropin. Furthermore, Engel notes (26) these properties are definitely not mediated

through the adrenal cortex since they are demonstrable in the adrenalectomized animal. The problem of the exact elucidation of those factors from the adenohypophysis affecting ketone and lipid metabolism is confounded by the fact that TSH also has fat mobilizing and ketogenic properties. In fact the thyroid stimulating hormone has been considered by some to be the adipokinetic and ketogenic hormone (26). Engel, however, believes that ACTH, TSH, and somatotropin each independently have ketogenic activity.

It seemed reasonable to assume that since the anterior pituitary secreted hormones that stimulated ketosis and lipemia and, further, that these effects were not mediated through the adrenal during the acute ketosis of hypoglycemia, it was the hypophysis that was the endocrine mediator of these effects. However, the hypophysectomized rat develops ketonemia almost as easily as the normal animal during and following hypoglycemia (55). Previously, it had been observed that ketosis of fasting did not supervene in the absence of the hypophysis. However, it was found that this was true only during a short fast. If the fast is sustained after 72 hours the hypophysectomized animal exhibits greater ketonemia than the intact animal (26).

Evidence has been cited above to the effect that ketosis occurs when the pituitary hormones are present in excess and also when they are deficient. This seems paradoxical. Engel (26) declares, however, that this is entirely in keeping with a basic tenet of endocrinology, i.e. that hormones do not initiate or stop metabolic reactions but only modify their rates. The profound hypoglycemia seen in the fasting hypophysectomized animal leaves the animal little choice but to draw on its lipid reserves which cannot be oxidized easily in the liver because of carbohydrate lack, a situation that favors ketogenesis. In the presence of adipokinetic principle on the other hand more lipid is again brought to the liver in excess of that which can be handled efficiently by the Krebs cycle; hence ketosis develops.

The Formulation of an Hypothesis

Before presenting the hypothesis an abbreviated scheme of intermediary metabolism should be reviewed. (Figure 1). Several features should be emphasized: Note that degradation of fatty acids involved successive beta-oxidation to acetyl CoA. Acetyl CoA then has several metabolic pathways available to it. Among these are:

- resynthesis of fatty acids

resynthesis of ketogenic amino acids

- cholesterol formation
- ketogensis
- condensation with oxalacetate to enter the Krebs cycle.

It is reasonable to assume that whenever any of these pathways are closed to acetyl CoA in states of deranged metabolism the other pathways will be accentuated.

Let us consider the metabolic situation in diabetes mellitus. ~~(Fig 2).~~ Because of block in the process of transfer of glucose across the cell membrane, a paradox of poverty and plenty supervenes; i.e., in extracellular fluid there is an abundance of glucose, while within the cell only a relatively meager amount available. This meager supply leads to a diminished rate of glycolysis and thereby a decreased availability of reduced DPN and FAD needed for synthesis of long chain fatty acids. Because of this, and possibly owing to a direct effect of insulin lack leading to decreased lipogenesis, the degradation of fatty acids to acetyl CoA is favored. The increase in formation of acetyl CoA is the fundamental feature leading to ketosis. It seems that the quantity of acetyl CoA exceeds the ability of the Krebs

cycle to accommodate it into oxidative pathways. Another feature seen in diabetes mellitus is the hyperlipemia. Since more fat is brought to the liver for oxidation from depots and since conditions in the liver favor catabolism of fat this, too, provides more acetyl CoA for accommodation into a Krebs cycle seemingly already overburdened. The result is further direction of acetyl CoA into other metabolic pathways. As mentioned above one of these pathways is that involving formation of ketone bodies.

I was interested in elucidating a gap in the scheme outlined above. Namely, how do the peripheral fat depots "know" that the organism is crippled with respect to carbohydrate metabolism ... i.e., what is the exact stimulus for the seemingly automatic mobilization of fat to the liver in states of carbohydrate deprivation? For purposes of this discussion carbohydrate deprivation is defined in a very broad sense. That is whenever the situation is such that carbohydrate is not being oxidized with facility through the metabolic pathway involving the Krebs cycle. The crippling of the efficiency by which carbohydrate is oxidized may take several forms. The most obvious one is an absolute lack of carbohydrate derived from exogenous sources, i.e.

starvation. Diabetes mellitus also falls into this broad classification of carbohydrate deprivation, because, as outline above, even though carbohydrate is ingested it is unavailable for normal oxidation because of its lack of availibility within the cell.

Similarly it has been observed that in glycogen storage disease, diabetic acidosis (56) as well as in starvation that there is hyperlipemia. All three of these, of course, involve profound derangements in carbohydrate metabolism.

It is an aphorism of modern intermediary metabolism that whenever there is a diminished rate of glycolysis, there is ketosis. It also appears that in the conditions mentioned above whenever there is a diminished rate of glycolysis (i.e. starvation, diabetic acidosis, and glycogen storage disease) there is a hyperlipemia.

The teleologist readily understands this state of affairs. He reasons that since the organism cannot use carbohydrate, the one prime fuel for its energy requirements, it turns to other sources, i.e. fat to supply its needs. This fat would have to come from the peripheral depots to be mobilized to sites for oxidation. Again the question arises, what signals the depots to mobilize fat? Ideally, this signal would be activated

automatically whenever a situation obtained in which fat was needed. Such a situation obtains whenever there is a diminished rate of glycolysis.

I submit that since ketones are formed in excess whenever there is a diminished rate of glycolysis that they would serve as an ideal signal to the depots to mobilize fat.

The physiologist may object to this line of reasoning here and say that the argument derives from teleological considerations. I submit that it is perfectly within reason to derive postulates for physiological mechanisms on teleological bases. Whatever the purist physiologist's objections to teleology as applied to the organism, one immutable fact is clear: THE ORGANISM SURVIVES. The reason that it survives is that most of its physiological interworkings perpetuate its survival. Therefore, it seems reasonable to say "this would be an excellent mechanism for the organism to invoke in order best to sustain itself." The proposal is then tested in the crucible of the experimental laboratory and is thus either upheld or refuted.

I hasten to point out that this hypothesis is based on a not inconsiderable body of information that links disorders of carbohydrate metabolism with ketosis and

hyperlipemia. In fact, one of the most prominent demonstrations of this triad of pathophysiology is the condition of diabetic acidosis, the metabolism of which has been outlined above. An obvious corollary of the hypothesis that ketones serve as a signal to the periphery to mobilize fat has its application to diabetic acidosis and for that matter in the ketosis of starvation; namely that KETOSIS BEGETS KETOSIS. The process may be explained as follows: The ketones formed as a by-product of a crippled rate of glycolysis signal the periphery to mobilize fat to liver. The depots respond and triglyceride is brought to the liver in excess of the latter's capacity to oxidize it. The more triglyceride brought to the liver for degradation the more acetyl CoA formed. The liver is already unable to accommodate the acetyl CoA already formed and the added lipid only accentuates its over-supply. Hence, more acetyl CoA condenses with itself to form more ketone bodies which are sent to the periphery. The increased output of ketones by the liver signals for more lipids to be mobilized, the latter come to the liver for degradation, more ketones are formed and so the process perpetuates itself.

Again, looking through the glasses of the

teleologist, this process should be looked upon as beneficial for the organism. There is a dire need for a source of energy since the preferred fuel, carbohydrate, is not utilizable. The ketones, by their subsequent oxidation by peripheral tissues provide an abundance of energy to the organism.

With the foregoing as a prelude let me formally state this hypothesis: THE KETONES FORMED AS A CONSEQUENCE OF A DIMINISHED RATE OF GLYCOLYSIS SERVE AS A SIGNAL FOR THE MOBILIZATION OF FAT FROM DEPOTS.

We are not prepared to speculate whether or not this signal effect is mediated by the hypophysis or any other endocrine gland.

In order to test this hypothesis sodium beta-hydroxybutyrate and acetone were administered parenterally into the rabbit and blood lipids measured at a predetermined time. If the lipids increased we felt this would lend support to the hypothesis. The experiment was not meant to test the validity of the corollary KETOSIS BEGETS KETOSIS.

METHODS AND MATERIALS

Housing and Feeding of Animals.

Thirty male New Zealand White rabbits were placed in cages made of metal with a coarse wire floor through which urine and feces could pass. Two animals were housed in each cage.

The animals were allowed a standard rabbit ration and water ad libitum provided in metal feeding dishes on the cage floor.

Initial Stabilization.

A young rabbit in good health gains weight rapidly. Although I have no precise data on the subject it was my impression from previous experiments that those rabbits who did not register a weight gain after one week on standard rabbit ration were probably diseased and would probably die within one week.

Since I was interested in measuring the effect of ketonemia in normal - not diseased - animals it seemed reasonable to invoke a screening test which involved the measurement of weight gain over a given period of time. The initial stabilization also allowed the rabbits to reach their own normal metabolic and behaviorial status that might have been disturbed secondary to shipment from the supplying rabbitry.

Blood Withdrawal.

Two sites of blood withdrawal were selected, the central artery of the ear and the left side of the heart. In either case a 5 cc. syringe was used with a one inch 20 guage needle. The rabbit was restrained in a wooden box especially built for this purpose allowing only the rabbits head to protrude. A goose-neck fluorescent desk lamp was placed nearby to insure adequate lighting. The rabbit's ear was heavily moistened with a toluene-soaked pledget and the ear rubbed briskly. The combination of the irritation produced by the toluene plus the rubbing caused a vasodilation that made the vessals easily visible. The artery was entered through the dorsum of the ear and the blood withdrawal accomplished. Four cc. of blood were withdrawn. Following the withdrawal of the needle we found it impossible to stop the flow of blood by direct pressure within a reasonable period of time. As excess blood loss might introduce another variable into these studies the practice was adopted of ligating the artery with silk thread. This measure effected prompt and adequate hemostasis. The procedure just described served to obtain the control or basal level of total lipids. The second sample was withdrawn by cardiac puncture in the

following manner. The rabbit was immobilized in a supine position and a needle introduced by the trans-thoracic route via the left chest. It was simple to ascertain whether or not the needle had entered the left side of the heart as we wanted it to by the color of the blood. Arterial blood in the rabbit, like in the human is bright red. If dark venous blood was inadvertantly obtained, the puncture was repeated until it was successful.

Preparation of the Ketone Mixture and Its Injection.

I did not know what concentration of ketones to use and in what proportion to body weight. A concentration of 10 per cent total ketones was finally decided upon arbitrarily. This figure is not without precedent. In 1937, Mirsky and Broh-Kahn (25) employed a 10 per cent solution of sodium beta-hydroxybutyrate which they injected intravenously into rabbits. It was felt that with this concentration we could inject enough total ketones into the animal without unduly diluting his total blood volume and still avoid using an unreasonable osmolarity.

There are two ways to inject the ketones. Instantaneously, and at a constant and sustained rate so as to reach a predetermined level of ketosis in a physiological manner. The instantaneous method has the obvious disadvantage of suddenly overloading the blood with a

very high concentration of ketones which would very rapidly be depleted by renal excretion, as well as by diffusion throughout body water. The instantaneous method was the one chosen only because it was technically easier. Its inherent errors will be discussed later as a possible reason for the results obtained in these experiments.

As was stated in the literature review, Mirsky and Broh-Kahn (25) found that 75 per cent of blood ketones are in the form of beta-hydroxybutyrate. Therefore it seemed reasonable to make up a solution for injection with 7.5 grams of sodium beta-hydroxybutyrate and 2.5 grams of acetone per 100 cc. Thus 75 per cent by weight of the solute of the 10 per cent solution was in the form of sodium beta-hydroxybutyrate and 25 per cent by weight of solute was in the form of acetone. Obviously it would have been better, in the interest of making a more physiologic solution, to add acetoacetic acid. However, this compound was not obtained in time for the experiment.

The pH of the ketone mixture was quite alkaline (circa pH 11) so to avoid introducing another variable, HCl was added dropwise to bring the pH to 7.4. The pH was measured with a carefully calibrated Beckman pH meter.

The ketone solution was injected into the rabbit in the following manner. With the rabbit immobilized in the animal restraining box the ear was prepared with toluene as for blood withdrawal. An ordinary paper clip was then placed on the lateral margin of the ear proximally so as to occlude the marginal ear vein. The occlusion with the clip resulted in engorgement of the vein facilitating entry of a 22 guage one inch needle on a 30 cc eccentric syringe. After entry into the vein no aspiration of the syringe plunger was needed. The blanching of the proximal part of the vein as the solution washed out the blood within the vessal was enough evidence that the needle was in place. Aspiration would have meant repositioning of the hands and an opportunity for the rabbit to shake the needle out of place. The injection was made as rapidly as the small-bore needle would permit.

Pilot Study No. 1

It was stated before that I did not know what amount of ketones to use in proportion to body weight. The optimal time for blood withdrawal to catch the peak of the anticipated lipemia was also unknown. Ideally ketones should have been injected and blood withdrawals performed at several predetermined intervals to outline

the complete trend of lipemia including the rise, the peak, and the return to normal levels of lipids. Unfortunately, I had no army of technicians and consequently had to limit my samplings to capture the optimal level of lipemia.

Accordingly, three rabbits were selected for the first pilot study. Ketones in the amount of 200, 400 and 800 miligrams per kilogram of body weight were chosen for injection. Blood samples were drawn at 0 time, 1, 2, 3, and 4 hours after injection of the ketones.

The method for the determination of serum lipids for all of these experiments was the of De La Huerga, Yesinick, and Popper (57). For the details of the method the reader is referred to the original article (57). However, the essence of the method is as follows: A small amount (0.5 cc) of serum is added to 9.5 ml of Bloor's mixture (one volume ethyl ether to three volumes alcohol) and the final solution heated for half an hour. Following this lipid extraction an aloquot of the mixture is taken and evaporated to dryness leaving the lipids which are then taken up by p-Dioxan, and the misture heated. After cooling the lipids are emulsified by the addition of sulfuric acid and the resulting

turbidity read in a spectrophotometer. For these determinations, one per cent triolein was used as a standard.

The results of the first pilot study are shown in Table I. Note that the rabbit who receiving 800 mg. of ketones per kilogram of body weight showed a marked increase (circa 65 per cent) in serum lipids over his basal level and that this increase was most pronounced four hours after ketone injection.

Pilot Study No. 2

I wondered whether or not injection of still more ketones per kilogram of body weight would cause the blood lipids to climb higher. Also I did not know whether or not the peak of lipemia might extend beyond four hours.

Four other rabbits were selected into whom were injected 600, 800, 1000, and 1200 mg. of ketones per kilogram of body weight. Blood was withdrawn at 0, 2, 4 and 6 hours. These results are found in Table II. Note that of these animals the one receiving 800 mg. per kilogram showed the greatest per centage rise in serum lipids. (about 46 per cent).

Since the lipid concentrations still seemed to be going up we decided to withdraw blood samples 50 hours

and 24 hours from pilot groups one and two respectively. The one rabbit in the first pilot study (code #5) showed a continued rise after 50 hours to .55 mg per cent total lipids. Those in the second pilot study showed only a slight tendency toward a rise after 24 hours.

Full Scale Experiment

From the information gained with the pilot studies, it appeared that the optimal amount of ketones injected would be 800 mg. per kilogram of body weight. The optimal time for blood withdrawal was another matter. No tendency for the serum lipids to drop even after 50 hours was recorded. However, it seemed that at six hours we were at least in the vicinity of the highest values we recorded over the 50 hours. Therefore, six hours for a second blood withdrawal seemed reasonable.

For the controls it was decided to use five animals into whom would be injected a solution of sodium chloride of the same osmolarity as the ketone solution, both of which were distincty hypertonic, being 1.6 osmolar.

Ten animals were in the experimental group into all of whom were injected 800 mg. of ketone solution per kilogram of body weight. The method of injection has already been described.

Blood samples were taken before the ketone injections

(time 0) and six hours later. With respect to the control group of five animals, the blood samples were also withdrawn at time 0 and six hours after injection of the sodium chloride solution.

Results

The results of the full-scale experiment are found in Table III. Note that of all of the animals six showed a significant rise in total serum lipids. Three of these were in the control group and three in the experimental group.

Discussion

Because of the fact that there was a rise in total serum lipids in the same number of animals in the control group as in the experimental group we can find no support for our hypothesis that ketones cause a hyperlipemia in the rabbit. THIS DOES NOT NEGATE THE VALIDITY OF OUR HYPOTHESIS. Just as one would have to be guarded in the conclusions that one might have drawn if the experimental group showed a significant rise of blood lipids over the controls so must one limit himself in the interpretation of this negative result.

Speculation as to why the experiment gave the results that it did are almost limitless. I could start with the idea that the rabbit may be just the animal that does not use the ketones as a signal to peripheral tissues

to mobilize fat. It could also be that a hypertonic solution per se - no matter what the nature of the solute - might initiate lipid mobilization in some but not in others.

Perhaps stress itself induced comparable changes in both the experimental and control groups. The possibility remains that the sampling was insufficient, i.e. more animals should have been used.

The mode of injection deserved special note. The fact that the ketones were injected instantaneously may have distorted the results. It is possible that they were so rapidly metabolized and/or excreted that whatever stimulus ketosis might have for lipid mobilization may not have been sustained long enough.

It may also be that a diminished rate of glycolysis imposes other modifications upon cells in general that sensitize them to the "signaling" effect of the ketone bodies. In the absence of such modification the signaling effect of the ketone bodies may not be operative.

Acetoacetate was not injected. This might be the very ketone body that is the effective one in signaling lipid mobilization. It may be that all three ketones must be present together to achieve our postulated effect.

For future experiments I would perhaps subject the animals to a short period of starvation to see if the exogenously added ketones would intensify the hyperlipemia. Other animals might be used. It is not unreasonable to consider using human subjects since the ketones are known to be physiologic substances. A constant infusion technique would certainly be more satisfactory than the instantaneous injection method. Also, the relationship of blood lipid rise to blood ketone concentration would further delineate the ketonemia-lipemia relationship.

Sometimes, when an investigator fathers an idea it truly becomes his "child". In so regarding it, the investigator nurtures it, protects it, and continues to prop it up against the force of arguments to the contrary. It is understandable that one abandons his ideas and theories with reluctance. I found myself in exactly this position as I started to terminate the discussion of why the experiment turned out as it did. I left out the mentioning of yet another possibility... ...that my hypothesis is wrong.

I submit, however, that it may have worth and deserves further investigation.

Summary

The literature on ketone bodies was reviewed with respect to their origin, site of production, blood levels, metabolism, and relationship to hormones.

An hypothesis was formulated placing the ketone bodies in the role of substances that signal the peripheral tissues to mobilize fat to meet energy needs. A special application of this hypothesis was made to the ketosis of diabetes mellitus.

The hypothesis was tested by injecting ketone bodies into rabbits and measuring the changes in serum lipids induced thereby. The results showed no significant changes in serum lipids over those induced in a control group. It was concluded that this experiment lent no support to the original hypothesis. It was pointed out, however, that this did not negate the validity of that hypothesis.

Experimental approaches other than the one used here were suggested to test further this hypothesis.

Figure I

An Abbreviated Scheme of Intermediary Metabolism

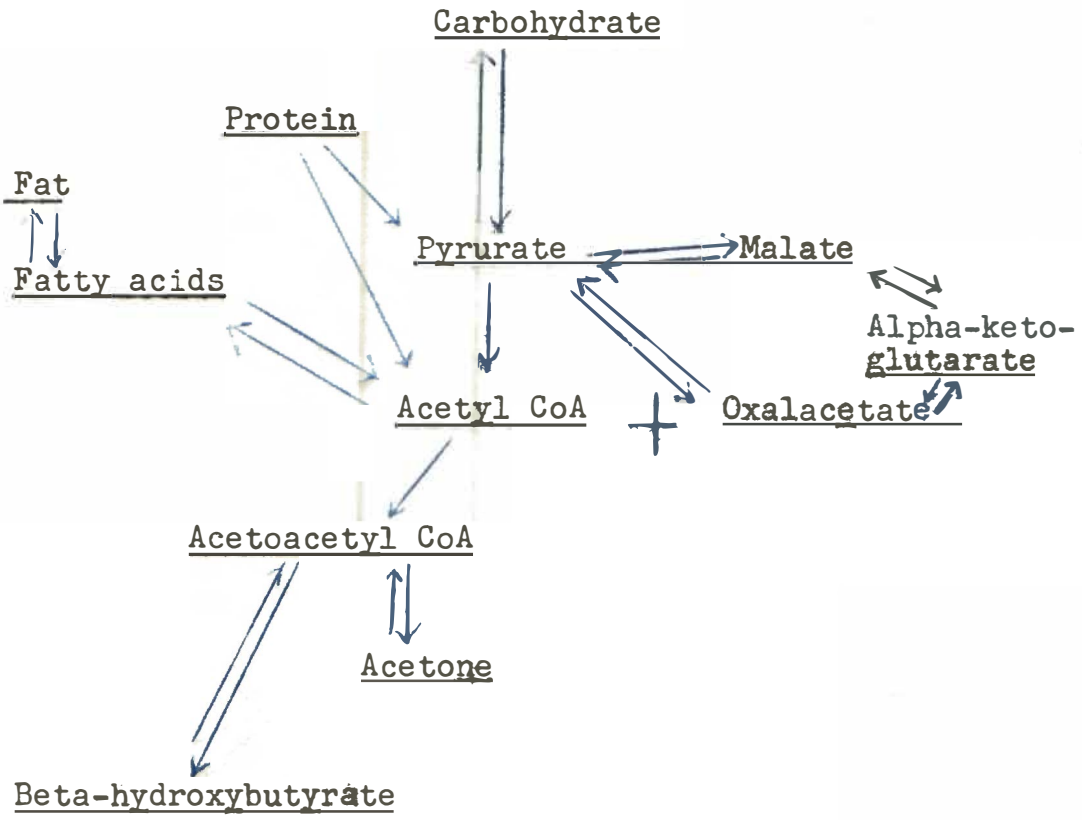

Table I

PILOT STUDY 1 - CHANGES IN TOTAL SERUM LIPIDS AFTER INJECTION OF VARYING AMOUNTS OF KETONES PER KILOGRAM OF BODY WEIGHT.

RABBIT CODE No	KETONES INJECTED (mg/Kg)	TOTAL SERUM LIPIDS (gm %) 0 time	1 hour	2 hours	3 hours	4 hours
A 1	200	.13	.14	.13	.15	.11
A 2	400	.18	.19	.12	.14	.14
A 5	800	.23	.24	.33	.33	.38

Table II

PILOT STUDY 2 - CHANGES IN TOTAL SERUM LIPIDS AFTER INJECTION OF VARYING AMOUNTS OF KETONES PER KILOGRAM OF BODY WEIGHT

RABBIT CODE No	KETONES INJECTED (mg/Kg)	TOTAL SERUM LIPIDS (gm %) 0 time	2 hours	4 hours	6 hours	24 hours	50 hours
A 9	600	.26	.22	.21	.23	.38	----
A 5	800	.23	.33	.38	----	----	.55
A 10	800	.28	.31	.27	.41	.44	----
A 6	1000	.36	.31	.31	.41	.42	----
A 2W	1200	.33	.35	.38	.44	.47	----

Table III

FULL SCALE EXPERIMENT

CHANGES IN TOTAL SERUM LIPIDS BEFORE AND AFTER INJECTION OF 800 mg/Kg OF BODY WEIGHT OF KETONES AND SALINE IN EXPERIMENTAL AND CONTROL GROUPS RESPECTIVELY

RABBIT CODE NO.	TOTAL SERUM LIPIDS (gm %)		PER CENT CHANGE
	0 time	6 hours	
Experimental Group			
B 1	.37	.41	+ 10 %
B 3	.35	.38	+ 8
B 4	.36	.34	- 6
B 5	.13	.14	+ 8
B 6	.46	.46	0
B 7	.20	.28	+ 40
B 8	.22	.20	- 9
C 6	.49	.78	+ 60
C 7	.37	.40	+ 8
C 10	.20	.35	+ 75
Control Group			
C 1	.59	.64	+ 8
C 2	.38	.40	+ 5
C 3	.32	.40	+ 25
C 8	.13	.19	+ 46
C 9	.25	.40	+ 60

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