Lilly Lecture 1978
New Perspectives in the Regulation of Ketogenesis

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In a review of the subject of ketogenesis and its regulation, Dr. Otto Wieland once stated that ". . . there is no other body constituent which may change in concentration in a dimension comparable to that of the ketone bodies."[1] It is hardly surprising, therefore, that the questions of *why* the body should produce acetoacetic and β-hydroxybutyric acids and *how* it controls their production have captured the attention of many investigators ever since these compounds were discovered in the urine of diabetics over a century ago. As to the first of these questions, the last 20 years have brought major advances in our knowledge of the role of the ketone bodies in the overall fuel economy of the body and have allowed a clear distinction to be made between the terms physiologic ketosis and pathologic ketosis. This aspect of ketone body metabolism has been dealt with in several recent reviews[2-4] and will not be discussed further. Rather, it is the purpose of this lecture to focus on a number of gaps in our understanding of the regulation of the ketogenic process itself. Prominent among these are the questions of which hormones exert primary control over ketone body production and how their effects are described in biochemical terms.

My introduction to this fascinating area of research came in January of 1968 when I had the good fortune of being accepted as a postdoctoral fellow into the laboratory of Doctor Daniel W. Foster, with whom I have had the honor and pleasure of working ever since. I should now like to review the sequence of events through which our own studies and thinking of this problem evolved during the past 10 years and, thereby, to place into historical perspective developments of recent months that promise to shed new light on the enigma of how the ketogenic process is controlled. I wish to acknowledge the efforts of Doctors Jurgen Meier, Carlos Robles-Valdes, Guy Mannaerts, and Yoichi Takabayashi, each of whom played an important role in our quest to unravel the long-standing mystery of mammalian intermediary metabolism.

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EARLY CONCEPTS

At the time we began our studies the prevailing view of events leading up to the development of the ketotic state can be summarized as follows: insulin deficiency → mobilization of fatty acids from adipose tissue → increased uptake and oxidation of fatty acids by the liver → accelerated production of acetoacetic and β-hydroxybutyric acids. It became increasingly evident, however, that this formulation was somewhat oversimplified. For example, it was shown that free fatty acids could be elevated in vivo without a major increase in the production of ketone bodies[5-7] and, conversely, that ketosis could be reversed in situations where plasma free fatty acid concentrations were maintained at elevated levels.[8,9] Taken together, these observations in vivo indicated that the rate of output of ketone bodies by the liver could not be dependent solely on the rate of input of fatty acids. This point was illustrated more directly in studies with the perfused rat liver, which revealed marked differences in acetoacetate and β-hydroxybutyrate formation for the same level of delivered fatty acids depending on whether the donor animal was fed, fasted, or diabetic.[10-14] Thus, it became apparent that, in addition to increased substrate availability, a fundamental change in liver metabolism was necessary to effect maximal rates of ketone body synthesis. Recognition of this fact posed a number of important questions, three of which became the major focus of our research. First, could we identify a primary regulatory site in the pathway of hepatic ketogenesis? Second, which hormones exerted control at this site? Third, how were the hormonal signals translated into biochemical events? What follows represents a summary of key developments as they emerged in our attempts to answer these questions.

LOCALIZATION OF THE REGULATORY SITE IN HEPATIC KETOGENESIS

The major disposal routes open to a long-chain fatty acid in liver are depicted in Figure 1. Several possibilities existed to explain the enhanced ketogenic response of livers from

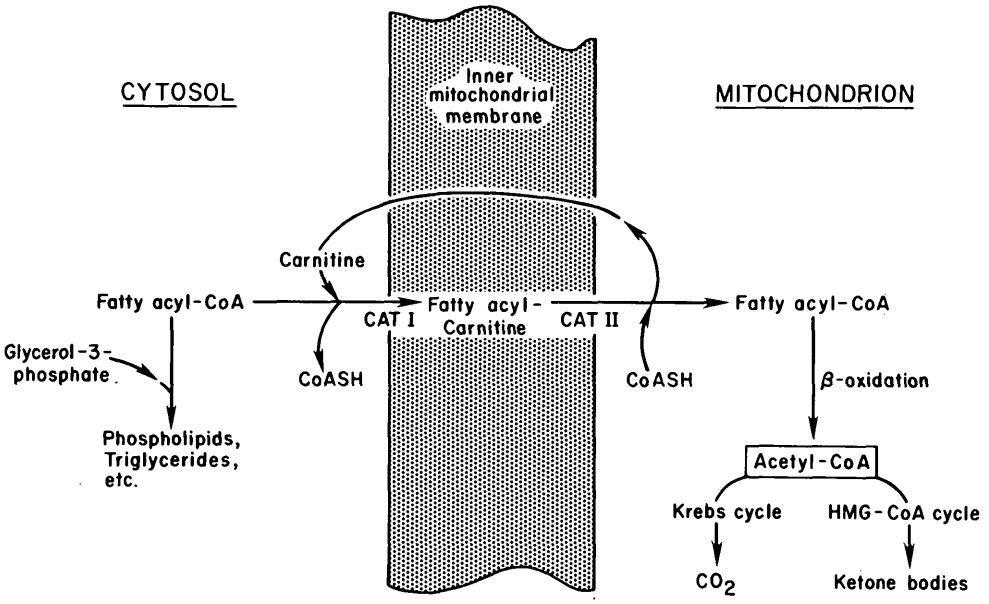

FIGURE 1. Major pathways of fatty acid metabolism in liver. CAT I and CAT II refer to carnitine acyltransferases I and II located on the outer and inner aspects of the inner mitochondrial membrane, respectively.

ketotic (fasted or diabetic) animals to a given load of fatty acid substrate. One was that ketosis might induce augmentation of one or more of the enzymes in the HMG-CoA cycle that catalyze the conversion of acetyl-CoA into acetoacetate. However, this notion became untenable with the demonstration that neither starvation nor diabetes substantially altered the rate of ketogenesis when freeze-thawed liver homogenates were incubated with saturating concentrations of acetyl-CoA.[15,16] It seemed, therefore, that, for a given input of fatty acids, the output of ketones by the liver must depend on both its ability to generate acetyl-CoA and its capacity to dispose of this compound through nonketogenic pathways. In this regard, it had been postulated that the accelerated production of acetoacetate in the ketotic liver might result from depression of Krebs cycle activity due to oxaloacetate deficiency[17] or direct inhibition of citrate synthase.[18] However, the validity of this theory was rendered questionable on the basis of studies, such as those from Regen and Terrell[19] and Williamson et al.,[20] which indicated that, in the perfused rat liver, depression of Krebs cycle activity was not essential for accelerated ketone body production. We addressed this issue in more detail by making a quantitative assessment of the metabolism of [1-^{14}C]octanoic acid and [1-^{14}C]-oleic acid through various metabolic pathways in perfused livers from fed, fasted, and alloxan diabetic rats.[13,14] In contrast to the physiologic long-chain fatty acid, the nonphysiologic medium-chain fatty acid is not used directly for triglyceride synthesis and does not require the carnitine acyltransferase (CAT) step for entry into the mitochondrion. The use of the latter substrate, therefore, allowed evaluation of events subsequent to the initiation of β-oxidation. In addition to providing further evidence against the primacy of the Krebs cycle in the control of ketogenesis, these experiments yielded another critical piece of information. In agreement with earlier studies by Mayes and Felts,[10] we found that, while all three types of liver took up similar quantities of oleic acid, the flow of fatty acid carbon through the β-oxidation and ketogenic pathways was greatly enhanced in the fasted and diabetic groups; in livers from fed animals, the low rate of oleate oxidation was balanced by a concomitant increase in the quantity of fatty acid entering the esterification pathway. In contrast, octanoic acid was oxidized at similar rates in fed, fasted, and diabetic livers with the result that differences in ketone production between normal and ketotic livers were much less pronounced than those seen with oleate as substrate.

These findings clearly indicated that primary control of the complete process of hepatic fatty acid oxidation and, thus, ketogenesis was vested in the partitioning of long-chain fatty acids between the pathways of esterification and oxidation. What remained to be established was whether the increased ketogenic capacity of the ketotic liver was a consequence of a diminished ability of the tissue to esterify incoming fatty acids, as originally suggested,[10,21,22] or the result of direct activation of the oxidative sequence. Although it was difficult to prove the operation of one of these mechanisms to the exclusion of the other, a number of observations pointed to the latter as the dominant event. First, there was a poor correlation between the ketogenic capacity of rat liver and the tissue level of glycerol-3-phosphate,[8,14] a compound previously considered to play a controlling role in the esterification system. Second, it was well known that livers from severely ketotic animals are invariably engorged with fat,[11,23] a finding difficult to reconcile with a fundamental defect in the esterification machinery of the liver under these conditions. Third, when livers from ketotic animals were perfused with oleate in the presence of D-decanoylcarnitine, an inhibitor of the carnitine acyltransferase reaction, ketone body production was abolished and essentially all the fatty acid taken up by the tissue was shunted into the esterification pathway.[24] In other words, blockade of the oxidative sequence caused a reversion in the pattern of fatty acid metabolism in ketotic livers to that seen in normal liver. It was observations such as these that led to the postulate[14,25,26] that primary control of hepatic fatty acid oxidation does in fact reside on the oxidative arm of the esterification-oxidation branch point. Moreover, in light of the differential effects of ketosis

TABLE 1
Effects of anti-insulin serum (AIS) and glucagon treatment of fed rats

Treatment	Plasma glucose (mg·dl^{-1})	Plasma FFA (mM)	Plasma ketones (mM)	Liver glycogen (mg·g^{-1})	Hepatic ketogenic capacity (μmol·100 g body wt^{-1}·30 min)
Controls	137 ± 12	0.54 ± 0.10	0.24 ± 0.02	31.6 ± 6.0	47 ± 2
AIS	438 ± 38	1.85 ± 0.13	1.51 ± 0.16	9.6 ± 2.0	154 ± 2
Glucagon	192 ± 21	0.37 ± 0.04	0.27 ± 0.02	3.8 ± 0.8	146 ± 8

Fed rats weighing approximately 100 g received an intravenous infusion of guinea pig serum containing 1.6–2.1 U of insulin antibody per milliliter, 100 μg of glucagon per milliliter or neither agent at a rate of 200 μl/min for 5 min, followed by 10 μl/min for 3 h. At this point the indicated analyses were performed. Hepatic ketogenic capacity was determined by measuring the rate of ketone production when livers were perfused with 0.7 mM oleate. Values are means ± SEM for four to eight animals in each group. The data are adapted from McGarry et al.[29]

on the hepatic metabolism of long- and medium-chain fatty acids (see above), we proposed that the actual control site was likely at the carnitine acyltransferase reaction, the first step specific to oxidation of long-chain fatty acids. Our working hypothesis, therefore, was that a key event underlying the enhanced ketogenic capacity of the liver in ketotic states was activation of this enzyme system.

HORMONAL FACTORS IN THE CONTROL OF HEPATIC FATTY ACID OXIDATION

We next turned our attention to the hormonal control of hepatic fatty acid oxidation. It was well established that a hallmark of the ketotic state is a reduction in the circulating level of insulin, a circumstance known to promote accelerated lipolysis in fat depots and increased delivery of fatty acids to the liver. What was not clear was whether the concomitant stimulation of the liver's capacity to oxidize these substrates was also the consequence of insulin deficiency per se. That this might not be the case was suggested by the fact that the counter-regulatory hormone, glucagon, whose concentration in plasma was known to rise in ketotic states, had been shown to have ketogenic activity in isolated liver preparations.[11,27,28] To examine the effects of glucagon in vivo, we carried out the experiment shown in Table 1. Short-term treatment of fed animals with anti-insulin serum (AIS) resulted in the expected rise in plasma glucose, fatty acids, and ketone bodies and depletion of liver glycogen stores. In contrast, infusion of glucagon, which also promoted the loss of liver glycogen, caused only a modest elevation of plasma glucose and no rise in plasma fatty acid or ketone levels. The key observation, however, was that livers taken from both AIS- and glucagon-treated animals exhibited equally marked augmentation of ketogenic capacity, reaching values close to those seen after 24 h of starvation. We interpreted the data as follows. The development of ketonemia in AIS-treated rats reflected the fact that both fatty acid mobilization from fat depots and hepatic fatty acid oxidation had been turned on. Under these conditions, glucose released from liver glycogen stores could not be disposed of peripherally, resulting in a profound hyperglycemia. In contrast, infusion of glucagon triggered hepatic glycogenolysis, which in turn led to a stimulation of insulin secretion from the pancreas secondary to the rise in blood glucose concentration.[30] As a result, glucose released from the liver was metabolized by nonhepatic tissues, adipose tissue lipolysis was suppressed, and, despite the enhanced ketogenic capacity of the liver, plasma ketones remained low because of the lack of availability of ketogenic substrate. Other experiments, not reviewed here, served to support the above interpretation.[29] The important lesson learned from these studies was that a "ketogenic" liver could be obtained from a nonketotic animal.

In reference to Figure 2, this situation occurs when circulating insulin levels are either normal or elevated such that site 1 is suppressed, but where glucagon excess is present to activate site 2. For ketosis to develop, both sites must be turned on. This requires insulin deficiency to

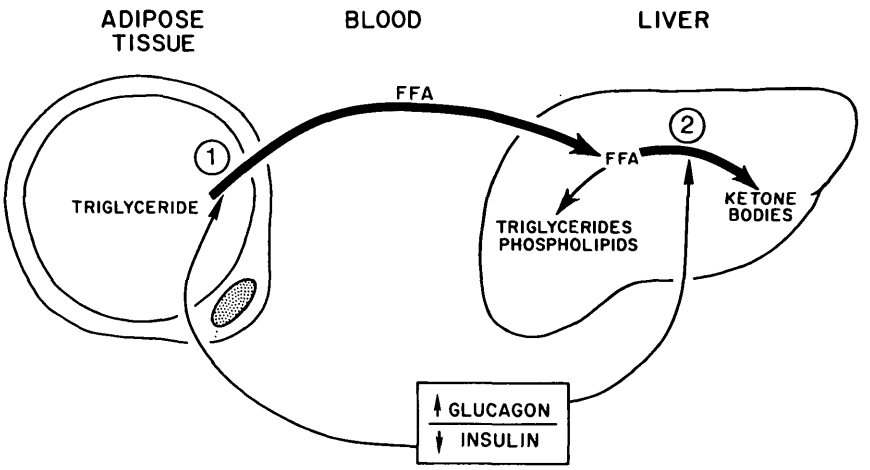

FIGURE 2. Bi-hormonal model for the control of hepatic ketone body production.

activate site 1; the concomitant hyperglucagonemia then serves as an important signal to turn on site 2. This is not to imply that insulin plays no role in the control of hepatic fatty acid oxidation. Indeed, it is likely that the glucagon: insulin ratio is the operative unit in the control of this parameter. While other hormones, particularly those associated with stress, undoubtedly play modulating roles at both sites 1 and 2, we believe that insulin and glucagon are the primary mediators through which rapid "on-off" regulation of the entire system is mediated. It thus appears that, in an analogous fashion to their roles in glucose homeostasis, as demonstrated by Unger and colleagues,[31] insulin and glucagon also constitute a bi-hormonal system for the control of the ketogenic process. Although this concept has emerged primarily from studies with the rat, evidence that similar mechanisms operate in other species, including the dog and man, is derived from the studies of Keller et al.,[32] Gerich et al.,[33] Alberti et al.,[34] and Schade and Eaton.[35] Furthermore, we believe that the model has explanatory power for understanding the several distinct types of ketosis (physiologic and pathologic) commonly encountered in clinical medicine (see ref. 4 for review).

MECHANISM OF HORMONAL CONTROL OF HEPATIC FATTY ACID OXIDATION

The final and most perplexing issue to be resolved had to do with how these hormonal signals are translated into concrete biochemical events that ultimately serve to govern the process of hepatic fatty acid oxidation. In light of our earlier studies, this question could now be rephrased more specifically—how does glucagon excess turn on the carnitine acyltransferase step? Available evidence suggested that alterations in the *activity* rather than the *amount* of this enzyme were responsible for regulating carbon flow through the β-oxidation pathway. If this were so, it followed that, in ketotic states, either a transferase-stimulating factor is generated within the liver or an inhibitor of the enzyme is lost; of course, a combination of both events was also possible. Consistent with the former mechanism was the finding that all manipulations we had previously employed to cause enhancement of ketogenic capacity in fed rats (acute treatment of animals with anti-insulin serum or glucagon, starvation, or treatment with alloxan) were also found to result in elevation of the liver carnitine concentration.[36] Furthermore, we observed that addition of carnitine to the medium perfusing livers from fed rats effected a marked stimulation of ketogenesis from oleate.[36] Since carnitine is a substrate for the carnitine acyltransferase I reaction, we concluded that an increased tissue concentration of this compound was an important element underlying the activation of hepatic fatty acid oxidation under conditions of glucagon excess. That it could not be the only factor emerged from a subsequent series of experiments on certain aspects of maternal and neonatal ketosis in rats.[37] This study revealed that, in the fed state, the lactating rat displayed a fourfold increase in hepatic carnitine concentration (i.e. similar to that seen in severely ketotic alloxan diabetic animals), and yet, when perfused with oleic acid, such livers showed only a modest enhancement in ketone production over that seen in livers from fed, virgin rats containing normal levels of carnitine. In seeking a solution to this paradox, we were struck by a unique feature of the lactating rat liver, namely, that in the fed state, high levels of carnitine coexisted with high levels of glycogen. You will recall that, in all our previous studies, any manipulation that resulted in acceleration of hepatic fatty acid oxidation was invariably accompanied by an *increase* in carnitine and *depletion* of glycogen stores of the tissue. We therefore concluded that both events were essential for maximal stimulation of the carnitine acyltransferase reaction.[4] Although a number of possibilities exist to account for the elevated hepatic carnitine levels of ketotic rats, the precise mechanism underlying this phenomenon has yet to be established. We were more intrigued by the significance of the inverse relationship between the glycogen content and the ketogenic capacity of liver. The final series of experiments was designed to investigate this phenomenon more thoroughly.

SEARCH FOR THE CARBOHYDRATE KEY TO THE CONTROL OF HEPATIC FATTY ACID OXIDATION

The notion that carbohydrate in some manner serves to suppress the oxidation of fatty acids in liver had been inferred from much earlier studies, such as those of Blixenkrone-Møller[38] and Mirsky,[39] and, in fact, led Chaikoff and colleagues to formulate the concept of "the carbohydrate sparing of hepatic fatty acid oxidation" in the early 1950s.[40] However, for the next quarter of a century, the mechanism of this interaction defied explanation. Our bias was that some metabolite, derived from glucose, whose concentration in liver fluctuates in parallel with that of glycogen, acts to suppress the activity of carnitine acyltransferase in the fed state. The question was which of the numerous intermediates operative in glucose metabolism might act in this capacity. Initially we considered that the putative inhibitor might be sought somewhere in the sequence of reactions leading from glycogen to pyruvate, in the pentose phosphate pathway, or perhaps in the Krebs cycle. To test this thesis, liver homogenates were incubated with radioactive oleate, ATP, coenzyme A, and carnitine, and rates of fatty acid oxidation were measured in the absence and presence of intermediates active in the above pathways. Despite an exhaustive search over a period of some 18 months, we failed to identify a compound capable of inhibiting the high rate of fatty acid oxidation in this system.

Faced with this dilemma we were forced to examine the problem from a wider perspective. Our reasoning ran along the following lines. A consistent finding in cellular metabolism is that opposing metabolic pathways are not usually simultaneously active. This had been well illustrated in the case of glycogen synthesis and degradation, in which at any given time the metabolic profile of the liver is set in such a way that the operation of one pathway takes preference over the other, thus avoiding massive futile cycling. In addition, it was recognized that specific factors responsible for activating glycogenesis also served to suppress glycogenolysis. It was attractive to suppose, therefore, that an analogous system might be operative in the control of hepatic fatty acid synthesis and oxidation, two processes that were also known to be affected in reciprocal fashion by changes in nutritional and hormonal status. Viewed from this standpoint, one compound active in fatty acid synthesis immediately sug-

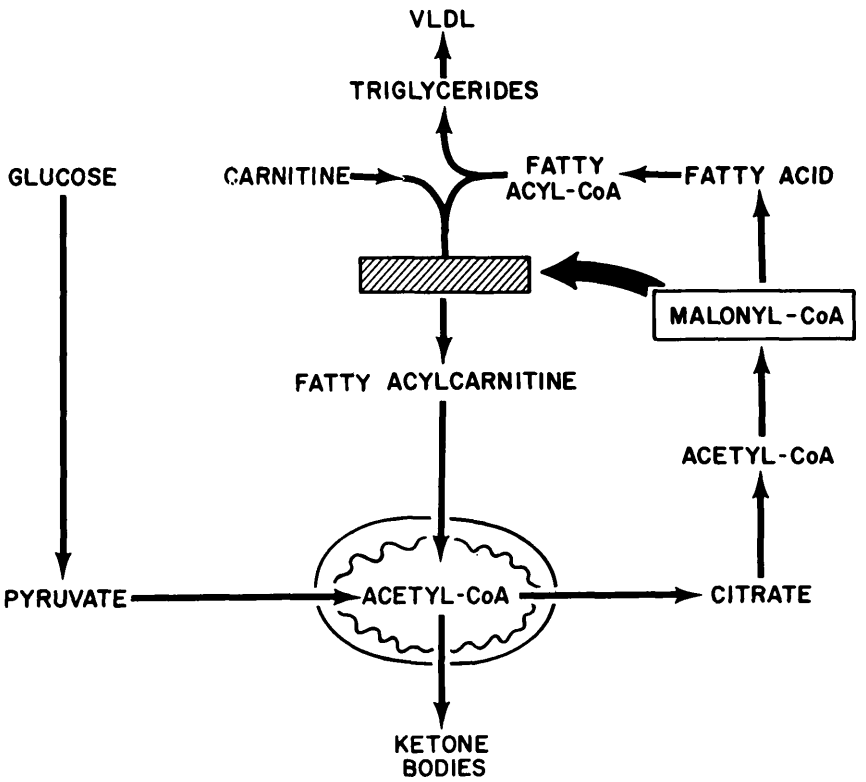

FIGURE 3. Interrelations between the pathways of fatty acid synthesis and oxidation in liver.

gested itself as a potential candidate to exert control over fatty acid oxidation in liver: this was malonyl-CoA. Our speculation was based on two considerations. First, malonyl-CoA represents the first committed intermediate in the conversion of glucose into fat. Second, its concentration in liver had been shown by Veech and co-workers to fluctuate in parallel with the rate of fatty acid synthesis, i.e. hepatic malonyl-CoA levels rose with carbohydrate feeding and fell precipitously with starvation or acute glucagon treatment.[41,42] Table 2 shows the first experiment in which we tested the effect of malonyl-CoA on the oxidation of oleate in a rat liver homogenate. At the time we arbitrarily chose a concentration of 0.5 mM and, to our gratification, found that this essentially abolished fatty acid oxidation under circumstances where 1 mM D-octanoylcarnitine, a known inhibitor of carnitine acyltransferase, produced only a 55% inhibition. The effect was shown to be specific for the malonyl ester of coenzyme A and to be readily reversible. Subsequent studies demonstrated that malonyl-CoA potently inhibited the mitochondrial oxidation of oleate, palmitate, and palmitoyl-CoA but had no effect on the oxidation of octanoate, octanoylcarnitine, or palmitoylcarnitine,[43,44] which suggested that its site of action was at the level of carnitine acyltransferase I, the suspected control site for hepatic fatty acid oxidation (see Figure 1). Other experiments using mitochondrial membrane preparations established that this is, in fact, the case and that malonyl-CoA acts as an extraordinarily potent competitive inhibitor of this enzyme, with an inhibitory constant in the region of 2 μM.[44] No other physiologic compound is known to be capable of inhibiting this reaction.

On the basis of these findings, we proposed the model depicted in Figure 3 to account for the reciprocal control of hepatic fatty acid synthesis and oxidation. Its key features are that, with carbohydrate feeding, the flow of glucose carbon to fatty acid is brisk, malonyl-CoA levels are elevated, and the tissue carnitine concentration is at its lowest. Under these conditions, carnitine acyltransferase I is suppressed and the opposing pathway of fatty acid oxidation is shut down. Thus, malonyl-CoA, the first intermediate specific to fat synthesis, serves to control the first committed step in fatty acid oxidation, thereby ensuring a unidirectional flow of carbon from carbohydrate → fatty acid → triglyceride → very low density lipoprotein. Conversely, in ketotic states, liver glycogen stores are depleted and the conversion of glucose into fat is markedly diminished, with the result that malonyl-CoA levels fall and the activity of carnitine acyltransferase I becomes derepressed. The concomitant rise in hepatic carnitine content produces maximal activation of the liver's capacity to oxidize fatty acids and thus to synthesize the ketone bodies.

TABLE 2
Effect of malonyl-CoA on the oxidation of oleate by rat liver homogenate

Additions	nmol [1-^{14}C]Oleate → product · 12 min^{-1}	
	CO_2	Ketones
None	0.92	94.0
D-Octanoylcarnitine (1 mM)	0.53	42.0
Malonyl-CoA (0.5 mM)	0.16	1.8

A whole-liver homogenate from a fed rat was incubated with [1-^{14}C]oleate bound to albumin, ATP, CoASH, carnitine, and the indicated additions. Under the experimental conditions used (see McGarry et al.[43] for details) ketone bodies were essentially the sole products of fatty acid oxidation.

We have recently tested the validity of the model in a series of experiments with isolated hepatocytes from fed rats.[45] In keeping with its predictions, we observed that, under widely divergent conditions of incubation, the pathways of fatty acid biosynthesis and fatty acid oxidation, when measured simultaneously, were related in a linear and reciprocal fashion. In addition, it was established that glucagon acutely switched the direction of fat metabolism from synthesis to oxidation. As expected, the greatest stimulation of fatty acid oxidation and ketogenesis was achieved when both glucagon and carnitine were added to the incubations. The ketogenic action of glucagon resided in the ability of the hormone to block the generation of malonyl-CoA from glucose through potent inhibition of glycolysis and partial suppression of acetyl-CoA carboxylase. The former effect undoubtedly stems from a cyclic AMP-mediated inhibition of pyruvate kinase and perhaps other steps in the glycolytic sequence.[46] Whether a similar mechanism operates in the control of acetyl-CoA carboxylase remains to be established. Consistent with the above observations, the lowest rate of fatty acid oxidation and highest rate of fatty acid synthesis occurred when cells were incubated with lactate plus pyruvate in the absence of glucagon. Most importantly, the cellular content of malonyl-CoA, which varied directly with the rate of fatty acid synthesis and inversely with the rate of β-oxidation, fluctuated through a range (1–6 nmol/g) entirely consistent with its postulated role in the coordination of these two central pathways of hepatic lipid metabolism.

CONCLUSION

It is almost axiomatic in science that no problem can ever be considered completely solved. While the question of ketogenesis and its regulation is no exception to this rule, I think it is fair to say that developments over the past decade have brought us much closer to a full understanding of this century-old problem. Available evidence now indicates that, while a number of organ systems and hormones are active, two organs—adipose tissue and liver—and two hormones—insulin and glucagon—play primary roles. As is evident from the foregoing discussion, our studies have focused principally on how the hypoinsulinemic-hyperglucagonemic state activates the liver's ketogenic machinery. Although we suspected as early as 1972 that a key effect of this hormonal imbalance must be activation of the carnitine acyltransferase step, which initiates the process of fatty acid oxidation, elucidation of the underlying mechanism proved particularly difficult. With our present advantage of hindsight I think we can now identify one factor, which, above all, constituted a major stumbling block to progress in this area. This was our tendency to view the process of hepatic fatty acid oxidation in isolation from the opposite, but equally important, process of hepatic fatty acid biosynthesis. It is now clear that the two systems are reciprocally controlled and that at any given time one will dominate over the other in response to changes in nutritional and hormonal status. Just as alterations in the circulating levels of insulin and glucagon have long been known to shift the direction of glucose metabolism in liver from storage to release, only recently have we recognized the fact that this same hormone couple also dictates the direction of hepatic fat metabolism. This appears to be effected by changes in the concentration of a simple compound, malonyl-CoA, which occupies a strategic position in the pathway of conversion of glucose into fat and has the unique ability to suppress the opposing pathway of fat degradation. While other factors might well be operative, it is attractive to suppose that malonyl-CoA represents a central component of the long-sought-after "carbohydrate key" to the control of hepatic fatty acid oxidation and ketogenesis.

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