Metabolism of Acetoacetate-3-C^{14} by Mammalian Heart Muscle. (22345)

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In a previous paper(1) we have presented evidence indicating that the net increase of cardiac glycogen observed in ketonemic animals(2) does not represent a direct conversion of ketone bodies *per se* to carbohydrate. That demonstration invalidates the most obvious interpretation which can be applied to the data of Lackey *et al.*(2), and relegates the ketonemia to some secondary role yet to be defined. While our data was direct and conclusive, the subject is one of sufficient importance to require evaluation from a different viewpoint before definitely ruling out the possibility that cardiac tissue may have some unique metabolic mechanism for dealing with ketone bodies.

In the present paper we have used the isolated dog heart to examine the metabolic path traversed by acetoacetate-3-C^{14} as reflected by the isotope distribution in the endogenous malate and succinate isolated at the end of the experiment to serve as an internal metabolic indicator. Glycogen is frequently employed in this capacity(3) but was valueless here because no tagging of that molecule occurs under the conditions used, an observation which is consistent with our *in vivo* experiments(2).

Materials and methods. The isolated dog heart was used in a simple glass apparatus containing the usual components and through which the heparinized blood (500 ml in all experiments) was circulated by means of a manual "rubber bulb-check valve" type of pump. The gas outlet from this closed system was led through 2 refluxing setups for trapping acetone in boiling acid mercuric sulfate (4) and thence to an efficient alkali CO_2 absorbing tower. A slight vacuum was used to overcome the mild resistance to the free flow of gas through the system. *Sodium acetoacetate-3-C^{14}* was synthesized and prepared by established methods(5). Five millimoles of the radioactive compound was introduced dropwise into the flowing blood just as it entered the marble filled gassing tower. The 500 ml of circulating fluid was thus 0.01 M with respect to acetoacetate. Our experiment made use of several different gases: 100% O_2, 95% O_2-5% CO_2 and, in a few instances, a gas mixture calculated to simulate the oxygen tension found *in situ* and composed of 19% O_2-5% CO_2-76% N_2. With the first 2 gases we used undiluted blood in the apparatus and obtained a very satisfactory heart preparation. With the latter mixture however, the blood did not appear well oxygenated and quickly assumed a marked viscosity with resultant limitation in coronary blood flow, and feeble heart action. In these instances we diluted the blood 1:1 with Ringer-bicarbonate in order to maintain an adequate fluid flow. Even then the preparation was not nearly as vigorous as at the higher oxygen levels. At the end of a 2 hour experimental period, 2 slightly different approaches were used to isolate the malate and succinate present in the heart tissue. In one instance we followed directly the procedure outlined by Needham(6) after adding 0.2 mM of succinate or malate carrier. In the other we introduced more drastic conditions to assure release of any tissue bound dicarboxylic acids, or those that might perhaps be tied up in some sort of ester linkage. Using a Waring Blender, the cardiac tissue was homogenized in ice water (carrier succinate added) and made to pH 2 with concentrated sulfuric acid. The entire material was steam distilled, collecting ten volumes of distillate, and the resulting tissue suspension reduced to an almost dry paste at mild temperatures using a blast of dry air. After adding concentrated sulfuric acid and dehydrating with plaster of Paris the succinate was recovered and purified(6). Occasionally we effected further purification by use of partition chromatography on silicic acid(7). Our results were not affected by the method of extraction. Carbon-wise degrada-

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TABLE I. Isotope Distribution in Malate and Succinate following Metabolism of Sodium Acetoacetate-3-C^{14} by Isolated Dog Heart.

Gas	Other additions	Total C^{14} added,* cts/min. (×1000)	$C^{14}O_2$ in respiratory CO_2†	% substrate to CO_2	Specific activity, malate cts/min. /mg/C Carboxyl C	Methylene C	Specific activity, succinate Carboxyl C	Methylene C
O_2	None	2,626	703,000	26.7	683	0		
19% O_2–5% CO_2–76% N_2	"	4,150	791,420	19	2,553	17	1,748	58
Idem	Adrenalin	4,150	337,777	8	2,026	10		

* As sodium acetoacetate-3-C^{14}.
† Blood acidified at end of experiment and bound CO_2 aerated using N_2.

tion of the isolated succinate or malate (and occasionally both) was effected by established methods(8) giving the activity respectively in the carboxyl and methylene carbons.

Results. According to current concepts, acetoacetate-3-C^{14} is metabolized by first splitting into 2 "acetate" molecules one of which, in this case, would be labeled in the carboxyl group. Further breakdown would be effected by traversing the tricarboxylic acid cycle and producing among the various other components, succinate and malate labeled exclusively in the carboxyl groups(3). For each "acetate" entering the cycle 2 carbons are lost as CO_2 and this mechanism therefore cannot account for a net increase in any component. If the results of Lackey *et al.*(2) represent a direct relation between ketone bodies and net carbohydrate synthesis, then a path not involving the tricarboxylic acid cycle must be invoked. This could happen, for example, if omega oxidation converted acetoacetate directly to oxalacetate and thence to glucose via pyruvate. In this instance 3 of the 4 initial carbons would be available for the net synthesis. Another possibility involves the reduction of acetoacetate to butyrate and thence to succinate via omega oxidation and finally to glucose as in the above case. The latter is unlikely in view of Lorber's(9) recent demonstration that butyrate is metabolized only in the conventional manner, an observation which we have confirmed in the isolated dog heart(10). If either of the latter mechanisms is operating on acetoacetate-3-C^{14} the endogenous malate and succinate would be tagged in the methylene carbons.

Table I gives data which are typical of our various experiments. Only comparison between the activity in the carboxyl *vs* methylene carbons should be made. The absolute specific activity of the several samples is not comparable because the amount of carrier dicarboxylic acid added to aid in the isolation procedure varied according to circumstances. It is obvious that in comparison to the carboxyl groups, the methylene carbons of the malate and succinate contain an insignificant amount of C^{14}. This is consistent with the view that acetoacetate is being metabolized only in the established manner, *viz*, by breakdown to two two-carbon fragments which then traverse the tricarboxylic acid cycle(3), a path which cannot effect a net increase of any component.

We have made additional observations that seem worth recording. We were curious to assay the possibility that the heart may contain dormant alternate metabolic paths on which it can draw in an emergency. Accordingly, we stimulated a preparation to abnormally vigorous activity by the periodic addition of adrenalin to the circulating blood. As shown in Table I the isotope distribution in malate was not altered. Whether the low total conversion to $C^{14}O_2$ (8%) is a function of the adrenalin or merely a reflection of variability from preparation to preparation is a matter under consideration.

Summary. The metabolic path of acetoacetate-3-C^{14} in the isolated dog heart has been determined using the isotopic distribution in the endogenous malate and succinate as an internal metabolic indicator. Isotopic carbon was found exclusively in the carboxyl

groups of malate and succinate as would be expected if acetoacetate was being utilized only by the established path (acetoacetate → 2 "acetate" → 4 CO_2 via the tricarboxylic acid cycle). This is taken as corroborative evidence for our previous conclusion that ketone bodies *per se* are not responsible for the net increase in cardiac glycogen noted in ketonemia.

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Effect on Cardiac Glycogen of Intravenously Administered Sodium Acetoacetate-3-C^{14}. (22346)

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In dogs made diabetic by pancreatectomy the glycogen content of liver and skeletal muscle decreases while that of heart increases (1). A similar pattern has been observed in rats injected with large amounts of acetoacetate or B-hydroxybutyrate(2). It becomes a matter of some importance to establish whether the ketone bodies *per se* are being metabolized in a manner directly responsible for this net increase in cardiac glycogen or are merely acting in some secondary role. In the former instance a new and yet unrecognized mechanism would have to be operating, since the conventional metabolic path (acetoacetate → 2 "acetate" → 4CO_2 via the tricarboxylic acid cycle) is not compatible with a net increase in any component. If ketone bodies on the other hand are acting only in some indirect manner, that role too must be defined. The data published by Lackey *et al.* (2) simply notes the quantitative increase of cardiac glycogen without throwing any light on possible mechanisms. The present paper is the first in a series designed to define this particular metabolic area. Here we have made the direct test of whether ketone bodies *per se* go to heart glycogen. We have injected into rats large amounts of acetoacetate-3-C^{14} and subsequently isolated the cardiac glycogen to determine the quantity and distribution of C^{14} in the glucose molecule. By this means strong inferences concerning the metabolic path can be obtained.

Materials and methods. Sodium acetoacetate-3-C^{14} was synthesized according to established methods(3) and put into a solution at pH 7.4 such that per ml there was 0.88 mM (11%) of acetoacetate containing an amount of C^{14} giving 600,000 cts/min. The quantity of isotopic material available limited the study to 3 rats. The nature of the results is such that valid conclusions are clearly possible despite the small number of animals involved. A 200 g rat, warmed by a heat lamp, was anesthetized with nembutal, the jugular vein exposed, and the fine cannula of an intravenous drip apparatus introduced. In each of our 3 experiments the gravity flow ceased after about 2½ hours at which time 3 ml (2.6 mM and 1,800,000 cts/min.) of the ace-

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