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I hereby recommend that the thesis prepared under my supervision by Norton A. Nelson

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Approved by:

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The Utilization of Acetone Bodies

I. The determination of acetone bodies in whole rats

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This report is from a dissertation submitted by Norton Nelson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry, University of Cincinnati.

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In studying the utilization of acetone bodies under a variety of experimental conditions, it became desirable to analyze whole animals and determine their content of exogenous or endogenous acetone bodies rather than depend upon the measurement of ketonemia or ketonuria. Several procedures for tissue analysis were attempted, but were subsequently discarded because they were either too time-consuming for the large number of determinations involved, or inaccurate.

For the determination of acetone bodies in blood, Barnes (1) modified the Van Slyke method (2) so as to permit the use of a sensitive iodometric procedure in the final measurement of the acetone precipitated by the Van Slyke procedure. We have adapted Barnes' modification to tissue analysis. The specificity, rapidity and convenience of this method warrants its general application. The present report details the method and apparatus employed in the studies to be reported in subsequent communications.

The preformed acetone, the acetone resulting from the hydrolysis of acetoacetate, and that resulting from the oxidation of B-hydroxybutyric acid are precipitated as complex Hg salts with Deniges reagent as in the original Van Slyke procedure. The precipitate is separated by filtration, washed and decomposed by a buffered hydrochloric acid solution, and the resulting acetone distilled into water, where it is estimated by the use of its reaction with iodine to form iodoform (3).

METHOD

Reagents:

20% copper sulfate: 200 gm. of $CuSO_4.5H_2O$ dissolved in water and made up to 1 liter.

10% calcium hydroxide: 100 gm. analytical grade $Ca(OH)_2$ suspended by thorough mixing in 1 liter of water. Must be completely resuspended by shaking before using.

50 vol. % sulfuric acid: 500 cc. of concentrated sulfuric acid diluted to 1 liter.

10% mercuric sulfate: 73 gm. analytical grade red HgO dissolved in 1 liter of 4N H_2SO_4.

5% potassium dichromate: 5 gm. $K_2Cr_2O_7$ dissolved and made up to 1 liter with water.

Combined precipitating reagent: 175 cc. 10% mercuric sulfate, 50 cc. of 50% H_2SO_4 made up to 250 cc. with water.

Hydrochloric acid - borate mixture: 38 gms. $Na_2B_4O_7.10\ H_2O$ dissolved in about 300 cc. hot water; cooled; 50 cc. conc. HCl added, and diluted to 1 liter.

40% sodium hydroxide: 400 gms. analytical grade NaOH dissolved in water and made up to 1 liter.

0.005 N Iodine: 25 cc. 0.1 N iodine and 15 grams potassium iodide made up to 1 liter.

0.005 N thiosulfate: 25 cc. 0.1 N $Na_2S_2O_3$, standardized against $KH(IO_3)_2$, diluted to 1 liter. Must be freshly diluted each day.

Apparatus:

The scale drawing Figure I is self-explanatory. The finger condensor has been found to be extremely efficient.

We have found that separation of Hg complex by centrifugation without loss is rather difficult. Filtration with a small filter stick, which is detached and left in the flask, has proven more rapid and convenient.

Two types of filter sticks have been used. (1) a glass tube

about 25 mm. long, with an outer diameter of about 6 mm. over 15 mm. of its length, has the last 10 mm. enlarged to receive a 7 mm. solid glass bead, preferably somewhat irregular, which serves as a base for an asbestos mat. (2) A second type, giving somewhat more uniform mats, has the same dimensions as the above, but has a lightly sintered glass wool mat about 7 mm. in diameter fused into the enlarged portion of the tube. The filter stick is attached by a short piece of rubber tubing to a bent glass tube, which is long enough to easily reach the bottom of the reaction flask, and which leads to a filter pump. The rubber tube should overlap the filter stick only 3 or 4 mm. so that at the completion of filtration it may be easily dislodged - after disconnecting the suction - by pushing it against the side of the flask. The mat is formed by dipping into a suspension of asbestos; after an even, dense mat about 1 mm. thick has been deposited, the filter is washed and is ready to use.

Satisfactory asbestos is prepared by making a suspension of medium fiber acid washed asbestos, so thin that the coarser fibers will settle to the lower 1/4 of a 2 liter cylinder in about 5 minutes. The supernatant is then drawn off and used without further dilution. When the bead type filter stick is used, a thick suspension is used to fill the large openings, after which a mat is formed from the thinner suspension.

Preparation of Tissue Extract:

The rat, killed by a blow on the head, is cut up rapidly into an ordinary meat grinder and ground up repeatedly. After the 4th grinding, a homogenous mixture of the rat is obtained, and aliquots can be taken for the determination. In all experiments, three aliquots of the minced tissue were taken in order to assure adequate sampling.

10 grams of the minced tissue are introduced into a 250 cc. volumetric flask containing 50 cc. 20% copper sulfate. This is conveniently accomplished by weighing the tissue on a tared piece of celluloid about $3\frac{1}{2}''$ X $5\frac{1}{2}''$ which is then rolled and inserted into the neck of the flask. The cylinder of tissue is then pushed into the flask with a footed glass rod and the celluloid and rod are rinsed with distilled water and withdrawn. The volume is brought up to about 190 cc. and the contents of the flask are mixed thoroughly and allowed to stand for 30 minutes. 50 cc. of 10% calcium hydroxide are then added, the flask thoroughly shaken and allowed to stand another thirty minutes. The mixture is diluted to 250 cc. mixed and filtered. The extract usually has a purple biuret tinge which in no way interferes with the subsequent analysis. This extract is both sugar and protein free.

Total acetone body analysis:

10 cc. of extract is measured into the reaction flask containing 4 or 5 small beads. 4 cc. of the combined reagent is added. The flask is placed in its support over a micro burner, a finger condensor inserted, and 0.1 cc. of water, roughly measured, placed in the space between the neck of the flask and the condensor. The flask heated at such a rate that the contents come to a boil in 2 to 3 minutes. Boiling is continued for about a half a minute to drive most of the air out of the flask, the burner is then removed and 0.5 cc. of 5% potassium dichromate are delivered into the space between the condensor and flask neck, and washed down with two portions of water, of about 0.1 cc. each. When most of the last washing has been drawn into the flask, heating is resumed, and the contents are evenly, but gently, refluxed for 90 minutes. The solutions are then filtered by suction through a filter stick, and the precipitate washed 2 or 3 times with about 5 cc. portions of water.

The solution should be filtered immediately after heating is stopped, for the reasons mentioned below. When filtration is completed, the filter stick is detached and left in the flask. This is a convenient point to interrupt the analysis, since the precipitate is quite stable.

B-hydroxybutyric acid only.

To 10 cc. of the tissue extract in a reaction flask, 0.3 cc. 50% sulfuric acid is added, and the level of the liquid carefully marked with a wax pencil. 10 cc. of water are added, and the solution boiled down to the marked volume. 2.8 cc. of 10% mercuric sulfate and 0.5 cc. of 50% sulfuric acid are then added, and the determination continued as above.

Decomposition of mercury acetone precipitate:

A 1" X 8" test tube containing about 10 cc. of water is fitted over the condensor delivery tube so that the tip of the latter is about 1/2 in. above the bottom of the test tube. To the flask containing the filter stick and precipitate, 10 cc. of the borate acid mixture are added; the flask is fitted to the condensor, and distillation started. Distillation, which should require 7 to 10 minutes, is continued almost to dryness, when the receiver is lowered to allow a few drops of distillate to rinse down the inside of the delivery tip. Meanwhile, its outside is rinsed with water.

An appropriate amount of standard iodine solution (preferably delivered just below the surface) is immediately added to the distillate followed by 5 cc. 40% sodium hydroxide. The contents of the tube are mixed with a footed glass rod (which serves through acidification and titration) and after about 10 minutes, the remaining iodine is released by slow acidification with 3 cc. of 50% sulfuric acid. In order to

avoid volatilization of iodine during addition of the acid, the test tube should be kept cool by immersion in a jar of cracked ice. The excess iodine is then titrated with dilute standard thiosulfate of similar titer to the iodine solution. 1% starch solution, of which 2 drops are added just before disappearance of the iodine color, is used as an indicator.

It is convenient to separate the blank due to reagents into two parts. The iodine consuming material arising from the reagents used during the precipitation and distillation - determined by carrying 10 cc. of water through the entire procedure, including filtration - is small and quite constant. The iodine consumption of the alkali and acid during the Messenger reaction is somewhat larger and more variable. Our practice is to titrate each day the amount of iodine to be used in the analysis, with the usual amounts of 40% sodium hydroxide and 50% sulfuric acid present, and to deduct from this the blank due to precipitation etc. (occasionally redetermined) then subtraction from this of the cc. of thiosulfate used in a given analysis, gives directly the cc. of standard iodine used by the acetone in that case.

Because of a slight solubility of the mercury complex in the precipitating mixture (more fully described below), very small amounts of ketones will not be detected by the above procedure. Amounts less than this minimal level which saturates the refluxing mixture can be estimated by adding a small known quantity of acetone or B-hydroxybutyric acid to the tissue extracts (Table 5).

Calculations:

Use of the equations based on analysis of standard solutions (see below) is best illustrated by an example; from tables 2 and 3.

$$\text{mgm. acetone} = 0.0518\ (I + 0.10)$$

mgm. B-hydroxybutyric acid = 0.112 (I + 0.06)

or

mM acetone = 0.000892 (I + 0.10)

mM B-hydroxybutyric acid = 0.00107 (I + 0.06)

and when a 0.4 gm. sample of tissue is taken:-

mM acetone/kilo = 2.23 (I + 0.10)

mM B-hydroxybutyric acid/kilo = 2.68 (I + 0.06)*

where I = cc. 0.005 N Iodine.

If in an analysis of 10 cc. of tissue extract representing 0.4 gm. of tissue, 2.03 cc. of 0.005 N iodine was used when carried through the total acetone body determination described above, and 10 cc. of the same extract when analyzed for B-hydroxybutyric acid used only 1.78 cc. of 0.005 N iodine, then 2.03 - 1.78 or 0.25 cc. of 0.005 N iodine represents acetone plus acetoacetic acid. Therefore:-

mM B-hydroxybutyric acid/kilo = 2.68 (1.78 + 0.06) = 4.93

mM acetone acetoacetate/kilo = 0.25 X 2.23 = 0.56

Total ketone bodies = 5.49 mM/kilo

The constant corresponding to the complex Hg precipitate held in solution is, of course, in this case applicable to the B-hydroxybutyric acid determination only.

EXPERIMENTAL

Proportionality of the Messinger reaction:

C.P. acetone dried over calcium chloride was redistilled; and

*The constant to be added to the cc. of Iodine in the above equation is a correction for the acetone-mercury complex which remains in solution. Since the same complex results regardless of the source of the acetone, the correction should be the same in either case. We use, as the probable value, the more reliable 0.06 cc.

the first and last fractions were discarded. 2 cc. were measured at 20° from a calibrated Van Slyke-Ostwald pipette into a two liter volumetric flask almost filled with water. When made up to volume, 1 cc. of this solution contained 0.792 mg. acetone. From this a dilute solution containing 0.317 mgm./cc. was prepared. The iodine equivalents of varying amounts of this solution (in a total volume of 20 cc. of water) were determined as described above.

As Table I demonstrates, the yield of iodoform (as shown by the iodine used) is proportional to the acetone taken over a quite considerable range. The slope, 0.0488 cc. 0.005 N iodine/mgm. acetone, of the line passing through the experimental points indicates an essentially complete reaction (99.2%) since the theoretical equivalent is 0.0484 cc./mgm. acetone. This proportionality evidently is maintained over a large range of iodine excess since no serious deviation was found with variation of the equivalent ratio of iodine to acetone, from 1.2 to 63.

Yield from varying amounts of acetone:

Varying amounts of acetone in 10 cc. of distilled water in reaction flasks were carried through the whole procedure as described in the method above, being boiled in the presence of dichromate for 1½ hrs. This, though unnecessary, duplicates the conditions of acetone precipitation in an actual determination. The results in Table 2, if plotted with the amounts of iodine used against the acetone taken, would show a straight line passing below the origin. This is shown by the constant in the equation, cc. Iodine = 0.612 (cc. acetone) - 0.102, fitted to these data by the method of least squares. This is a result of a slight solubility of the complex in the hot precipitation mixture, as will be described more fully in the case of B-hydroxy-

butyric acid. The yield as shown by the slope of the straight line, is about 94%, and is quite close to that obtained by Barnes (4).

Yield from varying amounts of B-hydroxybutyric acid:

The calcium zinc salt of synthetic B-hydroxybutyric acid was prepared according to Shaffer and Marriott (5). Calcium and zinc analysis of this salt gave the following results:

	Ca	Zn
Found	7.75% 7.76%	12.1%
Calculated for $CaZn(C_4H_7O_3)_4$	7.74%	12.6%

0.1990 gm. of this salt, equivalent to 0.1600 gm. of the acid was dissolved in 2 liters of water giving a solution containing 0.8 mg. B-hydroxybutyric acid in 10 cc. Varying quantities of this solution in a total volume of 10 cc. were analyzed as described above. The results are shown in Table 3. When the experiment was repeated substituting cold for hot filtration by cooling the flask in cold water to room temperature, from 0.06 to 0.09 cc. extra iodine were used. The equation fitted to the data secured by hot filtration is, mgm. B-hydroxybutyric acid = 0.112 (cc. 0.005 N iodine + 0.06). It is apparent then that the Hg complex is significantly soluble in the hot Deniges reagent; according to the constant in the equation, this amounts to an equivalent of about 6 gamma of B-hydroxybutyric acid. A solubility of similar magnitude was reported by Van Slyke (2). Hot filtration of the tissue extracts is made necessary by a contamination of the precipitate occurring on cooling. This cannot be completely accounted for by a blank obtained by oxidizing the B-hydroxybutyric

acid and boiling off the acetone before the precipitation is carried out.

This slight solubility of the mercury complex becomes of importance when one is dealing with very small amounts of acetone bodies. We have estimated levels within the solubility range, of the hot reaction mixture by adding a known amount of B-hydroxybutyric acid sufficient to saturate the solution. In Table 5 the results of several such experiments are shown, 0.1 cc. of calcium zinc B-hydroxybutyrate solution - containing 8.0 gamma of the acid - was added to each 10 cc. of tissue extract taken for analysis. In Table 5a are results secured by this technique on analysis of the mixed whole body tissue of 24 hour fasted female rats; in Table 5b are the values found by the same procedure with fed female rats.

The percentage yield of acetone from B-hydroxybutyric acid in the oxidation as determined from the slope of this equation and the previous acetone standardization, amounts to about 83%. This is higher than the 76% found by Van Slyke, as is also that reported by others (6). This variation in yield, that apparently occurs in the oxidation, makes it imperative to standardize the procedure with known amounts of B-hydroxybutyric acid if more than merely relative values are desired.

The elimination of acetone and acetoacetate by boiling the acidified solution causes no significant breakdown of B-hydroxybutyric acid as was shown when B-hydroxybutyric acid was subjected to the treatment used for elimination of acetone and acetoacetate. There was produced in one case a decrease of 2.5%, in another no change, in a third a decrease of 4.5%, and in a fourth, an apparent increase of 1.9%.

Recovery of B-hydroxybutyric acid and acetone added to tissue extracts:

Varying amounts of B-hydroxybutyric acid and acetone were

added to extracts of mixed whole rat tissue, prepared as described above, with the results shown in Table 5. Also rats were injected with known amounts of B-hydroxybutyric acid, killed, immediately ground and analyzed. These data indicate that added B-hydroxybutyric acid and acetone can be recovered quantitatively.

In Table 6 are shown the results of an experiment where B-hydroxybutyric acid in 0.5 cc. of water was intimately mixed with 10 gm. portions of ground whole rat tissue, and various intervals of time allowed to elapse before addition of copper sulfate. These data demonstrate that no significant disappearance of the B-hydroxybutyric acid occurs in the ground tissue under these conditions.

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Table I

Iodine Used by Standard Acetone Solutions in the Messinger Reaction

Number of Determinations	Standard Acetone Solution Taken* cc.	0.005 N Iodine Used cc.	0.005 N Iodine Calculated from Equation** cc.	Deviation (Calc)-(Found) cc.
10	0.1	0.062 (± 0.0028)***	0.055	0.007
2	0.2	0.135 (± 0.0035)	0.120	0.015
12	0.5	0.315 (± 0.0077)	0.314	0.001
12	1	0.631 (± 0.0071)	0.639	0.008
12	2	1.293 (± 0.0111)	1.288	0.005
2	3	1.940 (± 0.0071)	1.937	0.003
9	5	3.188 (± 0.0094)	3.235	0.047
4	10	6.500 (± 0.0128)	6.480	0.020

*1 cc. $\backsimeq$ 0.0317 mgm. acetone

**cc. Iodine = 0.649 (cc. acetone) - 0.010; or
mgm. Acetone = 0.0488 (cc. 0.005 N Iodine + 0.01); fitted to the data by the method of least squares

***Standard error the mean (SEM) = $\frac{\sqrt{d^2/n}}{\sqrt{n}}$

Table 2

Iodine Used by Varying Amounts of Acetone
(dichromate present, refluxed 90 minutes)

Number of Determinations	Standard Acetone Solution Taken* Average cc.	0.005 N Iodine Used cc.	0.005 N Iodine Calculated from Equation** cc.	Deviation (Calc)-(Found) cc. 0.005 N I
3	0.2	0.047 (±0.0054)***	0.020	0.027
3	0.5	0.230 (±0.0095)	0.204	0.026
8	1	0.503 (±0.0109)	0.510	0.007
4	2	1.083 (±0.0161)	1.122	0.039
10	5	2.933 (±0.0167)	2.958	0.025
7	10	6.039 (±0.0362)	6.018	0.021

*1 cc. ⥋ 0.0317 mg. acetone

**cc. Iodine = 0.612 (cc. Acetone) - 0.102; or
mgm. Acetone = 0.0518 (cc. 0.005 N Iodine + 0.10)
fitted to the data by the method of least squares.

***Standard error of the mean (SEM)

Table 3

Iodine Used by Varying Amounts of B-hydroxybutyric Acid (hot filtration)

Number of determinations	Calcium Zinc B-hydroxybutyrate Solution Taken* cc.	0.005 N Iodine Used	0.005 N Iodine Calculated from Equation** cc.	Deviation (Calc)-(Found) cc. 0.005 N I
3	0.1	0.023 (±0.0027***)	0.016	0.007
3	0.2	0.090 (±0.0082)	0.088	0.002
2	0.5	0.285 (±0.0106)	0.293	0.008
8	1	0.661 (±0.0173)	0.661	0.000
10	2	1.376 (±0.0075)	1.378	0.002
13	3	2.093 (±0.0256)	2.095	0.002
11	5	3.550 (±0.0294)	3.527	0.023
10	10	7.102 (±0.0577)	7.111	0.009

*1 cc. = 0.080 mgm. B-hydroxybutyric acid

**cc. Iodine = 0.717 (cc. std.) - 0.056; or
mgm. B-hydroxybutyric acid = 0.112 (cc. 0.005 N Iodine + 0.06)
fitted to the data by the method of least squares

***S.E.M.

Table 4

Recovery of B-hydroxybutyric Acid and Acetone Added to Tissue Extracts and Injected into Rats

Method of Addition	Ketones added mM/kilo		Ketones Recovered mM/kilo		% Recovered	
	B-hydroxy-butyric acid	Acetone	B-hydroxy-butyric acid	Acetone	B-hydroxy-butyric acid	Acetone
Added to Tissue Extract	8.69	3.15	8.72	3.02	100.3%	96.2%
			8.72	3.20	100.3%	101.6%
			8.29	2.99	95.4%	94.9%
Added to Tissue Extract	5.22	3.15	5.42	3.13	103.8%	99.3%
			5.34	3.16	102.3%	100.3%
			5.21	3.25	99.8%	103.2%
Injected into Rats which were killed immed-iately Ground & Analyzed	8.20		8.26		100.1%	
	8.31		8.39		101.0%	
	8.23		7.91		97.2%	
	8.68		8.35		96.2%	
	8.28		8.57		103.5%	

Table 5

Estimation of Low Ketone Levels by Addition of Small Known Amounts of B-hydroxybutyric Acid to Tissue Extracts

(a)
Reliability of Addition Procedure

B-hydroxybutyric acid, mM/kilo

Rat No.	1 Initial Analysis	2 Total after addition	3 Added	4 (2) - (3) = initial	% Calc. vs. Found
B6	.32	.48	.19	.29	91%
B7	.61	.92	.19	.73	120%
B8	.38	.58	.19	.39	103%
A33	.52	.74	.19	.55	106%
125	.41	.64	.19	.45	110%
126	.62	.79	.19	.60	97%

(b)
Results secured by Use of Addition Procedure With Fed Rats Receiving Glucose

B-hydroxybutyric acid, mM/kilo

Rat No.	1 Added	2 Total Found	(2) - (1) = initial	Average
145	.19	.23	.04	
146	.19	.17	.02	
147	.19	.18	.01	.02
148	.19	.19	.00	
149	.19	.25	.06	

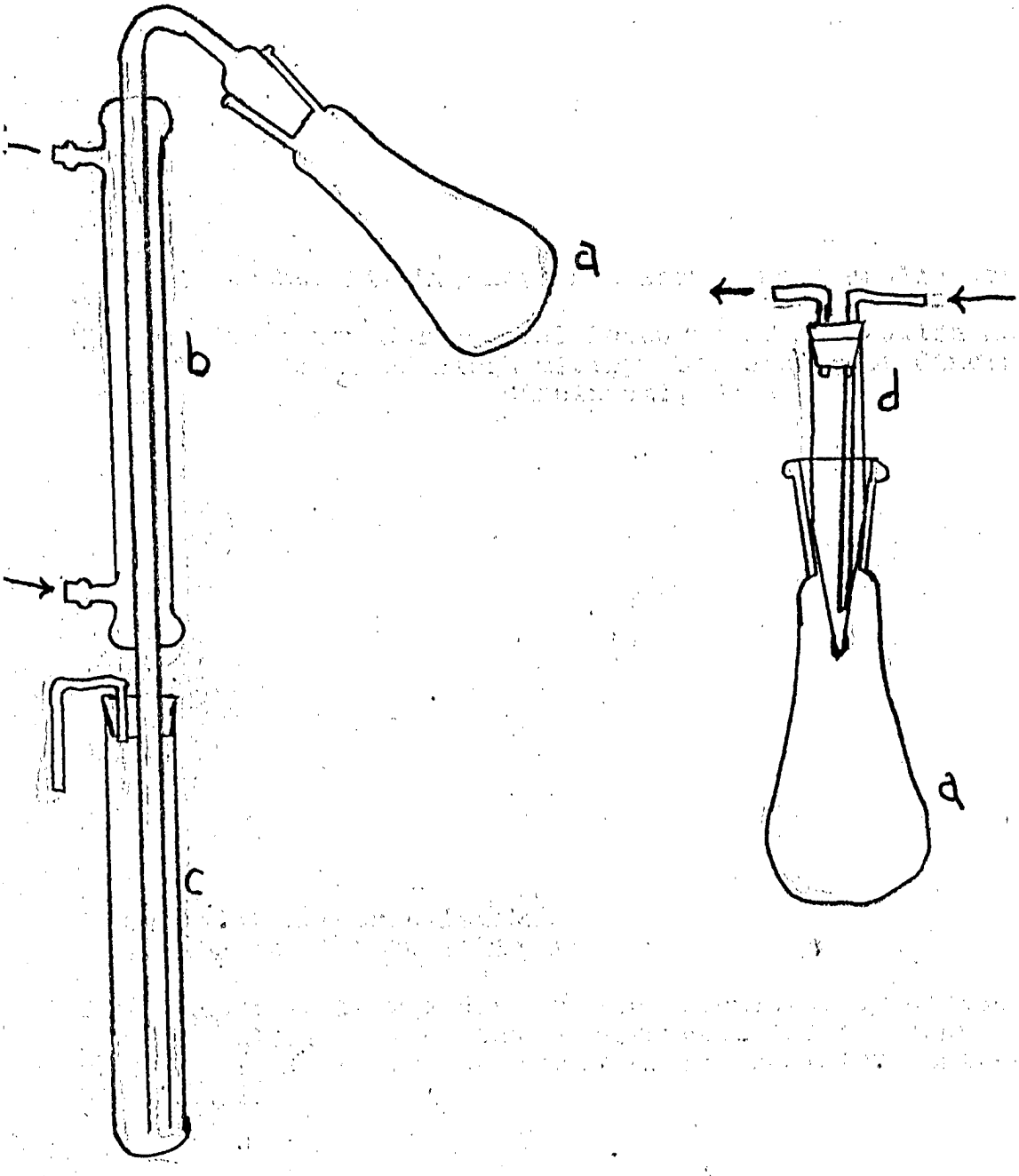
Table 6

The Effect of Fresh, Ground Rat Tissue on Added B-hydroxybutyric Acid

Time to addition of $CuSO_4$ minutes	Acetone mM/kilo	B-hydroxy-butyric acid mM/kilo
No tissue	0	8.46
0	0	8.52
20	0	8.38
80	0	8.42

LEGENDS

Figure I. Scale drawing of apparatus employed. (a) reaction vessel, 19/38 joint, (b) condensor used for distillation of acetone from mercury precipitate 19/38 joint, (c) test tube, (d) finger condensor, for refluxing, made from 15 cc. conical centrifuge tube.



The Utilization of Acetone Bodies

II The Influence of Feeding and of Glucose in Nephrectomized Female Rats

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This report is from a dissertation submitted by Norton Nelson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry, University of Cincinnati.

The production of ketone bodies generally is attributed to a decrease in the oxidation of glucose, while the oxidation of fat is concomitantly increased; the ketone bodies arising in consequence of an incomplete oxidation of the fat. This concept, first elaborated by Hirschfeld in 1895 (1) and subsequently epitomized by Rosenfeld (2) in the phrase, "fats burn only in the flames of carbohydrate" received extremely strong support from the "in vitro" studies of Shaffer (3). His observation that the disappearance of acetoacetic acid in the presence of hydrogen peroxide is accelerated when glucose is undergoing oxidation at the same time, was very suggestive of some chemical union between the acetone body and a product of glucose oxidation, It is such an increase in the oxidation of the ketone bodies which is referred to as the "ketolytic" effect of glucose.

This hypothesis provided basis for the assumptions that the ketone bodies are manufactured in all tissues of the body, that the diabetic organism cannot oxidize them and that the influence of carbohydrate in decreasing a ketosis is due to an acceleration of ketone oxidation (ketolysis). Contrary to these implications are the observations that the liver is the main, if not the only site of ketone body formation (4) (5); that the muscles of the diabetic organism can oxidize ketone bodies as readily as do those of the normal (6) (7); that the administration of glucose does not accelerate the rate of ketone body utilization by the muscles of normal rabbits (8) or by the perfused dog heart (9). However, the studies of Butts and Deuel (10), Shapiro (11), and more recently Deuel, Hallman and Murray (12) are not in accord with the conclusion that glucose exerts no ketolytic effect.

In view of the above, we considered it to be of some importance

to reinvestigate the problem concerning the influence of glucose, using some method which would take into account the procedures employed by Deuel and his associates. The method employed by these investigators consists in measuring the excretion of ketone bodies in the urine of rats after the ingestion of a definite amount of sodium acetoacetate. The difference between the amount fed and the amount excreted is interpreted to be the amount oxidized. Thus when Butts and Deuel observed that rats maintained on a stock diet excreted minimal amounts of the fed ketone bodies as compared with similarly treated fasted rats, or when Shapiro found that the administration of glycogenic metabolites resulted in a decrease in the ketone body excretion of rats fed diacetic acid, they concluded that carbohydrate produces an increase in the oxidation of the ketone bodies. The only consideration that these investigators gave to the possibility that changes in spontaneous endogenous ketogenesis might be a factor, was their observation that relatively small amounts of ketone bodies are excreted by control fasted rats. The probability that such fasted rats may be excreting only a small proportion of the total endogenous ketone bodies and oxidizing the remainder, received no obvious consideration. That this is an important consideration, may be gleaned from the fact that the excretion of acetone bodies in the urine is no indication of the concentration in the blood and tissues (13).

In order adequately to evaluate the above mentioned procedure and to determine whether glucose exerts a ketolytic action, we studied the actual disappearance of administered ketones from the entire bodies of nephrectomized rats during various periods of time. By investigating the acetone body content of control groups of rats, we could obtain a measure of spontaneous ketogenesis, and thereby make partial corrections in estimating the utilization of these substances under various experimental conditions.

METHODS:

This study was carried out on fed and fasted female rats from our stock colony. The fed groups consisted of animals which were kept on a stock-diet* until a few minutes before the beginning of the experiment, and which subsequently received an intraperitoneal injection of glucose. The fasted groups consisted of animals from which the food was removed twenty-four hours before the onset of the experiment. In order to prevent the excretion of injected ketone bodies, and also to obviate the effects of the kidneys 'per se', all animals were subjected to a bilateral nephrectomy under ether anesthesia. Immediately after the completion of the operation, the animals received an intraperitoneal injection of either saline or of a glucose solution in a dosage of 100 mgm. per 100 grams of body weight. After the animals recovered from the anesthesia, some groups of animals were treated as controls, while other groups received an intravenous injection of racemic sodium B-hydroxybutyrate. The dosage of the latter was kept as closely as possible to between 8.20 and 8.40 mM per kilo of body weight and was administered by means of a tuberculin syringe. At the completion of the experimental period, the animals were killed by a blow on the back of the neck, reweighed to ascertain the exact weight, thus establishing the exact dose of B-hydroxybutyrate that was administered, and then ground up in an ordinary meat grinder. The procedure employed for the determination of the ketone body content of the rat is described in the preceding communication (14), where its reliability is clearly indicated. All determinations were performed in triplicate.

The various experimental procedures may be best summarized as follows:

Group I. Fasted rats which were treated with saline and killed

*Stock-diet employed was purina fox chow.

at various intervals after recovering from the anesthesia.

Group II. Fasted rats which were treated with glucose and killed within 40 minutes after injection.

Group III. Fed rats which received glucose and killed within 40 minutes after the injection.

Group IV. Fasted rats which were treated with saline and killed 20 minutes after the intravenous injection of B-hydroxybutyrate.

Group V. Same as IV, but killed 40 minutes after injection.

Group VI. Fed rats which were treated with glucose and killed 20 minutes after receiving the B-hydroxybutyrate.

Group VII. Same as VI, but killed 40 minutes after injection.

RESULTS*

The results are detailed in Tables 1 to 9 in order to reveal the individual variations within the various groups. In the majority of the early experiments, the ketone body content of the animals was determined as B-hydroxybutyric acid, and acetoacetic acid plus acetone. However, we found that the average acetoacetic acid plus acetone fraction was 4.7% of the total, and, therefore, assuming a constant mixture of the various fractions, we performed single determinations and have expressed our results in all experiments in terms of mM of ketone bodies. The validity of this assumption is indicated by the fact that when the total ketones are estimated by summation of the individual fractions, the result is practically identical with that obtained by a single determination and the use of a suitable factor (Table I). As a check on this factor, frequent fractionations have been made throughout the study.

Table 2 reveals that an appreciable amount of acetone bodies

*Some of these results were reported in preliminary fashion in the Proc. Soc. Exp. Biol. & Med., 39, 51, 1938.

are found in fasted animals (Group I). However, the administration of glucose to such animals (Group II) results in a diminution in the acetone body content. In view of the considerations made in our previous communication (14), it is possible that, because of the small amounts involved, the actual content may be even lower than 0.20 mM per kilogram of body weight. This point is illustrated in Table 4, (Group III), where it is revealed that ketone body content of fed rats is equal to 0.02 when studied by addition experiments, and 0.20 when determined directly. It is evident from the data in Tables 3 and 4 that after the administration of glucose a lower endogenous acetone body content is found.

The rate of B-hydroxybutyric acid disappearance from the bodies of fasted rats during a twenty-minute period after the administration of B-hydroxybutyrate (Group IV), is detailed in Table 5, while that for the forty-minute period (Group V) is given in Table 6. Similarly, in Tables 7 and 8 are presented the rates for twenty and forty minute periods in fed, glucose treated rats (Group VI and VIII).

In Table 9, are summarized the data from all experiments, together with their statistical analysis. The latter clearly indicates that there is a very small dispersion of the data since the mean of each group is at least 10 times its standard error. Examination of this data reveals that if no accounting is made for endogenous ketosis, a small but statistically reliable difference exists between the utilization of B-hydroxybutyric acid by the fed rats receiving glucose, as compared with the fasted rats. However, endogenous formation of B-hydroxybutyric acid can be accounted for, though only in part, by adding the initial content of the rats to the amount administered, and then deducting from this total the amount of B-hydroxybutyric acid that is recovered at the end of the period. When this is done, the statistical

reliability disappears; decreasing from 5:1 to 2.3 in the case of the 20 minute groups and from 4.5 to 1.9 in the 40 minute groups. It is of interest to point out that if the probability that the glucose treated groups contain no acetone bodies is taken into account (Table 4) then the reliability of difference of those groups drops to 1.2 and 0.8 respectively. It must be emphasized again that these corrections account only for the acetone body content at the very outset of the experimental period, and not for the acetone bodies that are being produced during the remainder of the period. It is probable that the latter may account for the small difference that remains between the two groups even after the above corrections are made.

Another method for analyzing our data is a comparison of the rates of utilization between the twenty and forty minute interval groups of both fasted and fed rats. Such an approach obviates the necessity of correcting for the initial content, but not for the continued endogenous ketogenesis in the fasting rats. Thus we find that the difference between the fasting Group IV and V is 2.33 mM per kilo of body weight per 20 minutes, while that between the fed, glucose treated Groups VI and VII is 2.29 mM per kilo of body weight for the same interval of time. This indicates that there is no difference in the rates of utilization of B-hydroxybutyric acid between fed and fasted female rats.

DISCUSSION:

In a series of unpublished experiments we were able to confirm the observations of Shapiro and of Deuel and his associates that carbohydrate reduces the ketonuria which results from the oral administration of acetone bodies. However, the present investigation demonstrates very clearly that carbohydrate does not accelerate the utilization of acetone bodies by the extrarenal tissues of the female rat, and, there-

fore, is in complete contradiction to the conclusions reached by the above mentioned investigators. It is probable from our data that the reduction in ketonuria observed by these investigators is not a reliable index of acetone body oxidation, but is probably due to some other factor. Furthermore, our data are in accord with the results obtained from studies on normal and eviscerated rabbits and on the perfused heart, and validate the conclusions derived from those studies.

The administration of adequate amounts of carbohydrate results in the prevention or cessation of ketosis in human subjects (1), in phlorhizinized dogs (15) and in fasted, completely depancreatized dogs receiving no insulin (13). Since the ketone body content of the blood and urine is dependent upon the difference between the rates of ketone body formation and utilization, any reduction in ketosis consequent to such treatment must be due to either an increase in the rate of ketone utilization (ketolysis) or to a decrease in the rate of ketone formation (antiketogenesis). The data herein reported indicate that glucose is not ketolytic in the sense defined and hence supports the concept that antiketogenesis is the mechanism whereby carbohydrate brings about a diminution in ketosis (e.g. Group II). Accordingly, the decreased excretion of ketone bodies observed by Deuel et al is probably due to reduction of the endogenous ketogenesis induced by resting.

It is generally acknowledged that the liver is the main, if not the only site of ketone body formation, and that ketogenesis always occurs when the liver glycogen falls below some critical low level. Conversely it is also known that the restitution of the liver glycogen content to approximately normal levels by the administration of glycogenic substances results in a cessation of ketosis. Furthermore, the evidence obtained by Quastel and Wheatley (16), by Snapper and Grunbaum (17), and from our studies with rabbits, indicates that the ketone

bodies probably are not oxidized further in the liver, thus suggesting that these substances are end-products of fatty acid oxidation in that organ. Therefore, it is probable that the antiketogenic action of glucose is due to a cessation of fattyaacid oxidation in the liver in consequence of the glycogen that becomes available for phosphorylation and oxidation. This hypothesis, that glycogenic substances substitute for fatty acids and other ketogenic materials in supplying energy to the liver, is in accord with the theory of substrate competition for available oxygen, as suggested by Edson's (17) observation that fatty acids compete with other oxidizable substrates for the oxidizing systems of the liver. It is also in accord with the observations of Shapiro that only glycogenic substances are antiketogenic. The fact that Deuel, et al could demonstrate no effect with ethyl alcohol, is in agreement with the fact that this alcohol is not glycogenic and suggests that the acetic acid which may be derived from the oxidation of ethyl alcohol in the liver, like the acetone bodies themselves, is not utilized by the liver, but is secreted into the circulation to be utilized by the muscles (18).

SUMMARY:

If a correction be made for endogenous ketogenesis (initial acetone body content), it is observed that the utilization of intravenously injected B-hydroxybutyrate by nephrectomized female rats is not influenced by fasting or feeding or by the administration of glucose.

Carbohydrate does not exert a ketolytic action, and, therefore, its effect in abolishing ketonuria is due to a sparing of fat or other ketogenic substances, i.e., antiketogenesis.

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Table I

Comparison of Total Acetone Bodies Estimated by the Determination of the Fractions, B-hydroxybutyric acid and Acetoacetic Acid Plus Acetone, with the Total Acetone Bodies Estimated by a Single Determination and the Use of a Suitable Factor.

Rat No.	Separately Determined			Total Ketones Estimated with Factor (2.66 X (Iodine used + .06) from single determination	Deviation of calculated total ketones from summated fractions
	BOH mM/K	Acetoacetic acid plus acetone mM/K	Total ketones mM/K	mM/K	%
1	4.91	.56	5.47	5.55	1.3
2	5.28	.24	5.52	5.55	0.5
3	5.59	.54	6.13	6.21	1.0
4	4.75	.20	4.95	4.96	0.2
5	5.44	.00	5.44	5.41	0.6
6	2.76	.22	2.98	3.01	0.7
7	2.50	.04	2.54	2.52	0.8
8	2.77	.19	2.96	2.98	0.7
9	2.67	.10	2.77	2.77	0.4
10	3.20	.18	3.38	3.42	1.2
11	5.93	.43	6.36	6.40	0.5
12	6.45	.09	6.54	6.56	0.2
13	5.50	.08	5.58	5.56	0.4
14	5.67	.00	5.67	5.63	0.7
15	5.88	.24	6.12	6.14	0.2
16	3.18	.36	3.54	3.59	1.1
17	3.48	.16	3.64	3.64	0.0
18	4.04	.21	4.25	4.27	0.2
19	4.34	.01	4.35	4.32	0.9
20	2.68	.11	2.79	2.79	0.0

Table 4

The Acetone Body Content of Fed Nephrectomized Female Rats

(Group III)

Rat No.	Weight	m M BOH/K added to aliquot	m M BOH/K found	actual initial ketone content m M BOH/K
14	194	0	0.19	
19	142.5	0	0.18	
49	136	0	0.24	
76	163.5	0	0.22	
77	147	0	0.19	
81	157.5	0	0.33	
82	155	0	0.17	
99	163	0	0.17	
100	149	0	0.16	
101	152	0	0.22	
Av.	157.0	0	0.21	
145	191	0.19	0.23	0.04
146	188	0.19	0.17	-0.02
147	196	0.19	0.18	-0.01
148	219	0.19	0.19	0.00
149	166	0.19	0.25	0.06
Av.	192	0.19	0.20	0.02

Table 2

The Acetone Body Content of Twenty-Four Hour Fasted Nephrectomized Female Rats

(Group I)

Rat No.	Weight	m M BOH/K found
16	215.5	0.34
64	132	0.45
73	151	0.88
74	150.5	1.35
75	142.5	0.91
78	133	0.98
79	143	0.44
80	120.5	0.83
91	127	0.27
92	138	0.24
105	139	0.41
107	137	0.45
108	125.5	0.30
117	120.5	0.81
118	124	0.58
119	159.5	0.28
120	140.5	0.50
124	159	0.32
125	146.5	0.48
126	166.5	0.75
Av.	143.5	0.58

Table 3

The Influence of Glucose on the Acetone Body Content of Twenty Hour Fasted Nephrectomized Female Rats

(Group II)

Rat No.	Weight	m M BOH/K found
102	147	0.24
103	133	0.21
104	153	0.21
106	124	0.18
111	140.5	0.14
112	135	0.29
113	134	0.16
114	139	0.14
115	126	0.22
116	153	0.21
Av.	138.5	0.20

Table 5

The Utilization of B-hydroxybutyrate During a Twenty-minute Period by Twenty-Four Hour Fasted Nephrectomized Female Rats

(Group IV)

Rat No.	Weight	m M BOH injected per kilo	m M BOH/K recovered	m M BOH/K used
3	145	8.37	6.40	1.97
17	138.5	8.29	6.51	1.78
22	141.5	8.35	5.56	2.79
23	121	8.40	5.63	2.77
25	132	8.45	6.14	2.31
27	133	8.14	5.88	2.26
34	167.5	8.23	6.87	1.36
36	146	8.32	7.47	0.85
39	147	8.26	6.40	1.86
41	149.5	8.33	6.53	1.79
121	162.0	8.30	6.08	2.22
122	133.5	8.34	6.42	1.92
123	163.5	8.23	5.62	2.61
127	132	8.20	7.32	0.88
128	140	8.20	6.34	1.86
129	150	8.09	6.08	2.01
130	143	8.72	7.53	1.19
131	146.5	8.73	7.08	1.65
132	161	8.35	6.83	1.52
133	152.5	8.17	6.27	1.90
Av.	145.0	8.33	6.45	1.88

Table 6

The Utilization of B-hydroxybutyrate During a Forty-minute Period by Twenty-Four Hour Fasted Nephrectomized Female Rats

(Group V)

Rat No.	Weight	m M BOH injected per kilo	m M BOH/K redovered	m M BOH/K used
4	165	8.15	3.59	4.56
12	181	8.15	3.64	4.51
15	158	8.30	4.27	4.03
18	177	8.34	4.32	4.02
24	139	8.26	2.79	5.47
26	153.5	8.33	4.00	4.33
30	116	8.20	4.23	3.97
35	167.5	8.42	4.70	3.72
38	139	8.02	4.41	3.61
40	135.5	7.99	4.20	3.79
52	154	7.88	3.65	4.23
53	149.5	8.12	3.36	4.76
54	159	8.03	3.40	4.63
55	123	8.27	4.08	4.19
56	131	8.26	4.27	3.99
83	124	8.20	4.64	3.56
84	139	8.26	4.71	3.55
85	133	8.38	3.60	4.78
86	132	8.20	3.83	4.37
88	144.5	8.17	4.04	4.13
Av.	146.0	8.20	3.99	4.21

Table 7

The Utilization of B-hydroxybutyrate During a Twenty-minute Period by Fed Nephrectomized Female Rats Receiving Glucose

(Group VI)

Rat No.	Weight	m M BOH injected per kilo	m M BOH/K recovered	m M BOH/K used
5	161	8.36	5.55	2.81
7	179	8.25	5.55	2.70
9	183.5	8.40	6.21	2.19
13	123	8.27	4.96	3.31
20	148	8.20	5.41	2.79
29	140	8.67	6.78	1.89
32	139	8.73	6.52	2.21
46	145	8.59	6.18	2.41
110	147.5	8.23	5.64	2.59
134	145	8.59	5.50	3.09
135	167	8.44	5.05	3.39
136	186.5	8.44	6.04	2.40
137	138.5	8.53	5.92	2.61
138	150.5	8.50	6.02	2.48
139	132.5	8.42	5.67	2.75
140	184	8.38	5.72	2.66
141	146	8.53	5.90	2.63
142	139	8.26	5.11	3.15
143	133	8.38	6.39	1.99
144	136.5	8.89	6.44	2.45
Av.	151.0	8.45	5.82	2.63

Table 8

The Utilization of B-hydroxybutyrate During a Forty-minute Period by Fed Nephrectomized Female Rats Receiving Glucose

(Group VII)

Rat No.	Weight	m M BOH injected per kilo	m M BOH/K recovered	m M BOH/K used
6	151	8.47	3.01	5.46
8	162.5	8.28	2.52	5.76
10	178	8.48	2.98	5.50
11	160	8.41	2.77	5.64
21	140	8.44	3.42	5.02
28	130	8.08	4.42	3.66
31	148.5	8.39	3.18	5.21
33	136.5	8.89	4.77	4.12
42	154	8.31	3.56	4.75
44	137	8.38	3.91	4.47
45	129	8.39	3.93	4.46
47	148	8.42	3.69	4.73
48	151	8.25	3.03	5.22
50	171.5	8.22	3.81	4.41
51	132.5	8.17	3.56	4.61
93	149.5	8.56	3.62	4.94
94	149	8.58	3.22	5.36
95	133.5	8.60	3.45	5.15
96	146	8.53	3.51	5.02
98	159.5	8.23	3.44	4.79
Av.	148.0	8.40	3.49	4.91

Table 9

Summary of Experiments on the Utilization of Intravenously Administered B-hydroxybutyrate by Nephrectomized Fed and Fasted Female Rats

Experimental Group	No. of Rats	Body Weight grams	BOH Injected mM/kilo	Time after BOH injection	BOH found mM/kilo	B-hydroxybutyric Acid* Utilization	
						Uncorrected for initial content mM/K/interval	Corrected for initial content mM/K/interval
Group I Fasted, Saline Treated	20	143.5	0	--	0.58 * (± 0.065)	--	--
Group II Fasted, Glucose Treated	10	138.5	0	--	0.20 (± 0.014)	--	--
Group III Fed, Glucose Treated	10	157.0	0	--	0.21 (± 0.015)	--	--
Group IV Fasted, Saline Treated Plus Ketones	20	145.0	8.33	20	6.45 (± 0.118)	1.88 (± 0.118)	2.46 (± 0.135)
Group V Fasted, Saline Treated Plus Ketones	20	146.0	8.20	40	3.99 (± 0.105)	4.21 (± 0.105)	4.79 (± 0.123)
Group VI Fed, Glucose Treated Plus Ketones	20	151.0	8.45	20	5.82 (± 0.087)	2.63 (± 0.087)	2.84 (± 0.088)
Group VII Fed, Glucose Treated Plus Ketones	20	148.0	8.40	40	3.49 (± 0.116)	4.91 (± 0.116)	5.12 (± 0.117)

Reliability of Differences**

Groups	Uncorrected for initial ketone content	Corrected for initial ketone content
20 min. Groups IV and VI	5.1	2.3
40 min. Groups V and VII	4.5	1.9

*Standard error of the mean

$$S.E.M. = \frac{\sqrt{d^2/n}}{\sqrt{n}}$$

**Reliability of Difference = $\frac{\text{Difference of means}}{\text{Standard error of difference}}$,

Standard error of difference = $\sqrt{(SEM_1)^2 + (SEM_2)^2}$