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144. Shigeharu Tanayama and Michio Ui : Determination of Small Amounts of Ketone Bodies in Blood.

(*Faculty of Pharmaceutical Sciences, School of Medicine, Hokkaido University*[*1])

Inasmuch as the significance of ketone bodies as intermediaries in lipid metabolism has been the subject of a wide investigation, various methods have been worked out for quantitative determination of these substances in body fluids. Most of the methods previously described were based upon the conversion of 3-hydroxybutyric and acetoacetic acids to acetone and the subsequent determination of the acetone thus formed by gravimetric,[1] titrimetric[2~4] or colorimetric[5~9] procedures.

These methods, however, could not be applied to routine assay in which a number of simultaneous determinations were required, because the distillation process employed in most of the methods for isolation of acetone not only necessitates the presence of relatively large amount of ketone bodies in reaction mixtures, but also tends to give variable values as a result of complicated techniques. Though some investigators[5,7] succeeded in determining the oxidation product as 2,4-dinitrophenylhydrazone in the presence of oxidizing reagent without distillation, the inevitable extraction of color product formed another technical complexity.

Recently, Bessman and Anderson,[10] in their preliminary communication, suggested the possibility of performing oxidation, diffusion and color development with salicylaldehyde-acetone reaction in standard screw-capped test tube without any transfer until colorimetry. Their publication, though without any experimental procedures, urged the present authors to study the salicylaldehyde method with an aim to establish so a sensitive and simplified method as to be adopted for routine assay of ketone bodies in small amount of body fluids.

The principle of the method to be presented consists in the conversion of 3-hydroxybutyric and acetoacetic acids into acetone and the determination of the latter by colorimetric technique. The entire procedure, except for the colorimetry, is carried out as one-unit process in a 9×120 mm. glass-stoppered pyrex tube without any distillation or extraction needed in most previous methods.

Methods

Commercially available analytical-grade reagents were used in this study, except acetoacetic acid which in the form of its Ba salt, was prepared according to the directions of Meyer[11] as follows :

Five grams of ethyl acetoacetate was mixed with 80 ml. of 2.6% KOH solution and was kept at 0° for 48 hr. The hydrolyzate after adding 20 ml. of $2N H_2SO_4$, extracted from the solution with Et_2O

*1 Kita-12-jo, Nishi-5-chome, Sapporo, Hokkaido (棚山薫晴, 宇井理生).

1) G. Deniges : Compt. rend. Acad., **126**, 1868 (1898).
2) T.E. Weichselbaum, M. Somogyi : J. Biol. Chem., **140**, 5 (1941).
3) R.H. Barnes, A.N. Wick : *Ibid.*, **131**, 413 (1931).
4) R.S. Hubbard : *Ibid.*, **49**, 375 (1921).
5) L.A. Greenberg, D. Lester : *Ibid.*, **154**, 177 (1944).
6) W.L. Bloom : J. Lab. Clin. Med., **51**, 824 (1958).
7) E.E. Werk, *et al.* : Invest., **34**, 1256 (1955).
8) G.D. Michaels, *et al.* : *Ibid.*, **30**, 1483 (1951).
9) J.B. Lyon, W.L. Bloom : Can. J. Biochem. Physiol., **36**, 1047 (1958).
10) S.P. Bessman, M. Anderson : Federation Proc., **16**, 154 (1957).
11) V. Meyer : Chem. Ber., **15**, 1327 (1882).

which was distilled off under reduced pressure. The reaction product (free acid) was then rubbed with 10 g. of $BaCO_3$ in 80 ml. of distilled H_2O, following which the excess of $BaCO_3$ was filtered off. The aqueous solution of barium acetoacetate was freed from unreacted starting materal by shaking with Et_2O four times and the H_2O was removed by freeze-drying.

Results

In this method, as noted above, 3-hydroxybutyrate and acetoacetate are converted to acetone by heating in a boiling water bath with acid dichromate, and the acetone formed, after adding of reasonable amounts of sodium sulfite (to destroy the excess of $K_2Cr_2O_7$) and potassium hydroxide (to neutralize H_2SO_4), is submitted to condensing reaction with salicylaldehyde in strong base (potassium hydroxide) to form 1,5-bis(2-hydroxyphenyl)-3-pentadienone, the color of which is measured by colorimetric procedure. A variety of factors influencing these reactions are examined here, attempting to raise the sensitivity as highly as possible.

1. Time Necessary for Color Development

a) Effect of temperature : Time course of the color development at various temperatures is shown in Fig. 1. To 1.0 ml. of acetone sample (5γ/ml.) in a test tube, 0.5 ml. of 1% salicylaldehyde (in 3*N* KOH) and 1.0 ml. of 50% potassium hydroxide (w/w) were added. The solution was diluted with 3.0 ml. of distilled water before colorimetry. The blank was prepared by using 1.0 ml. of distilled water instead of acetone solution. At the higher temperature in the shorter time a plateau was reached, whereas the degree of the color development was higher in lower temperature. Therefore color development at 0° for more than 16 hours was adopted in the standardized procedure.

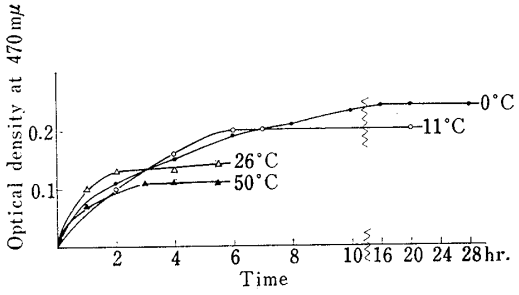

Fig. 1. Time Course of Color Development at Four Different Temperatures
Each value shows the mean of two observations.
For detailed procedure, see text.

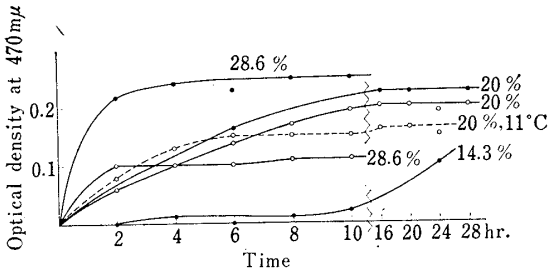

Fig. 2. Time-Development Curve in Various Concentrations of Potassium Hydroxide
•—• : In the absence of oxidizing reagent
o—o : In the presence of oxidizing reagent
Figures shown in percentage denote the KOH concn. in the reaction mixture.
Values show means of two observations.
The reaction was carried out at 0°, except where noted.

b) Effect of potassium hydroxide concentration : The concentration of potassium hydroxide to be added also exerts a profound effect on the time of the reaction, as shown in Fig. 2. To the mixture of acetone solution (5 γ) and 1% salicylaldehyde was added various amounts of potassium hydroxide The solution was diluted with distilled water to give a total volume of 5.5 ml. before reading. Higher concentration of potassium hydroxide gave good results in respect to the time required and the color intensity produced. For example, when 28.6% of potassium hydroxide (2.0 ml. of 50% potassium hydroxide was used in the reaction mixture, the period as short as 2 hours. was satisfactory for the maximum development of color. In the presence of oxidizing reagent, similar relationship was noted, but in this case a total volume of the solution never

failed to increase to keep the potassium hydroxide concentration at 28.6% (5.0 ml. of 50% potassium hydroxide was added) because 50% of potassium hydroxide was nearly its saturated concentration. As the increase in a total volume caused the decrease of the color level as a result of dilution, 20% of potassium hydroxide (2.5 ml. of 50% potassium hydroxide) was employed in this method. Under this condition, though the reaction was rather timeconsuming, the final color intensity was not so inferior to the maximum level obtained with 28.6% to potassium hydroxide in the absence of oxidizing reagent.

2. Stability of Color

In some methods previously described,[6,14] the reaction product was diluted with water before reading. The result shown in Fig. 3. illustrates, however, that the color formed diminished rapidly after adding water. It was ascertained that this color fading was not due to the decrease of potassium hydroxide concentration in the solution because the similar tendency was noted with 20% potassium hydroxide. Process of dilution was omitted in the present method, for, as shown in Figs. 1 and 2, the color intensity was maintained for several hours once attained to its maximum level.

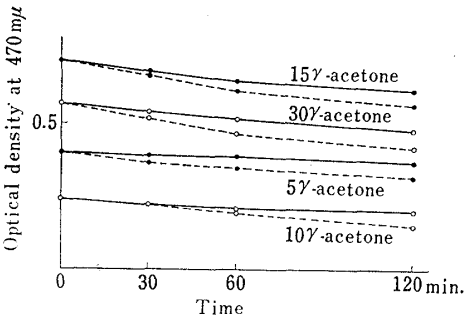

Fig. 3. Stability of Color after Dilution
•—• without oxidizing reagent : diluted to give a total volume of 5.5 ml. before reading.
○—○ with oxidizing reagent : 3.0 ml. of H_2O (or 20% KOH) was added to the reaction mixtures.
—— diluted with H_2O
------ diluted with 20% KOH

3. Conversion of Acetoacetate and 3-Hydroxybutyrate to Acetone

a) Optimum concentrations of sulfuric acid and potassium dichromate in oxidizing reagent for the oxidation of 3-hydroxybutyric acid : The yield of acetone from 3-hydroxybutyric acid by oxidation was studied with various concentrations of sulfuric acid and dichromate at 100° for 60 minutes. When the concentration of sulfuric acid in 0.5 ml. of the oxidizing reagent was below 6*N*, the yield was not satisfactory, though it was somewhat compensated for by raising the concentration of potassium dichromate (Table I) The similar result was reproduced in another series of experiments. Therefore, 0.2% solution of potassium dichromate in 6*N* sulfuric acid was employed as oxidizing reagent in this method.

Table I. Effect of Dichromate and Sulfuric Acid Concentration in the Oxidizing Reagent on Oxidation of 3-Hydroxybutyrate

$K_2Cr_2O_7$ (%)	H_2SO_4 Concentration (*N*)			
	2	3	6	9
0.1	0.033	0.063	0.128	0.137
0.2	0.063	0.097	0.145	0.133
0.3	0.085	0.107	0.136	0.120
0.4	0.109	0.128	0.140	0.115
0.5	0.109	0.136	0.129	0.104

Figures expressed as optical density denote means of two observations. The condition of color formation : 0°, 20 hr.

14) P. W. Krog, J. C. Lund : Acta Physiol. Scand., **12**, 141 (1946).

b) Time of heating for the oxidation of 3-hydroxybutyrate: The time required for the oxidation of 3-hydroxybutyric acid was investigated at 100° (Fig. 4). acetone solution was also treated in the same way, to examine whether any part of acetone was lost in the heating process. As shown in Fig. 4, the oxidation of the 3-hydroxybutyric acid was completed in 60 minutes, during which period little amount of acetone was lost.

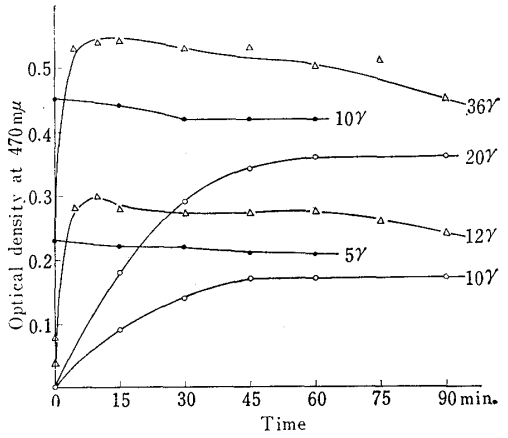

Fig. 4. Effect of Heating Time on Color Development
○—○ : 3-hydroxybutyric acid
•—• : acetone
△—△ : acetoacetic acid
The color development was carried out at 0° for 20 hr.

c) Conversion of acetoacetic acid to acetone : Ten minutes' heating was satisfactory for the decarboxylation of acetoacetic acid to acetone, as presented in Fig. 4. In the method reported by Greenberg and Lester,[5] the conversion of the keto acids to acetone was carried out as a single step with acid dichromate. On the other hand, Michaels, *et al*[8] insisted that this one-step method resulted in the poor recovery of 3-hydroxybutyric acid and proposed to perform these reactions in two steps, adding sulfuric acid and potassium dichromate separately. These two methods were compared in each case of acetoacetate and 3-hydroxybutyric acid (Table II); the serum was also included to test the practical usefulness of both methods. Two-step process was performed as follows :

Table II. Comparison of Two Methods in the Conversion of Keto Acids

Sample	Acetoacetate		3-Hydroxybutyrate	
γ/cc.	A	B	A	B
10	0.132	0.186	0.170	0.178
20	0.265	0.374	0.365	0.374

Sample	Serum (total ketone bodies)			
	I		II	
	A	B	A	B
Blood level mg. (%)	7.3±0.08 (7)[a]	7.6±0.06 (6)	7.0±0.15 (7)	6.9±0.20 (7)

A : One-step method B : Two-step method
Figures in the case of acetoacetate and 3-hydroxybutyrate present optical density and are means of two observations.
a) Mean ± S.E.M. : figures in parentheses is No. of observations.

One ml. of sample to be analyzed and 0.25 ml. of 12*N* sulfuric acid were successively put in the glassstoppered tube and they were heated at 100° for 10 minutes. After cooling, 0.25 ml. of 0.4% potassium dichromate was added to each tube, following which the heating was repeated at the same temperature for 60 minutes. As shown in Table II, the two-step method had advantages over the one-step method as to conversion of acetoacetate to acetone, but there found only slight differences bet-

ween oxidation reaction of 3-hydroxybutyrate proceeding in both procedures. The values of ketone bodies found in fasted rat serum was scarcely influenced by the separation of heating step as evident in this Table, presumably because the serum level of acetoacetate was low compared to other components.

4. Color Reagent (Salicylaldehyde)

a) Effect of the concentration of the color formation: When relatively small amount of acetone (5γ/ml.) was tested, the concentration of salicylaldehyde as 1% was satisfactory to produce the maximum color reaction (Table III). At the higher level of acetone in the test solution, however, optical density further increased with the rise of concentration of the color reagent. The 2% solution of salicylaldehyde was adopted in the present method, because salicylaldehyde by itself showed maximum absorption at 470 mμ, the same wave length as that for reaction product, resulting high blanc values in the series of higher concentration of the reagent.

Table III. Effect of Concentration of the Salicylaldehyde Reagent

Acetone concn. (γ/cc.)	Salicylaldehyde % (0.5 cc.)		
	0.5	1.0	2.0
5	0.093	0.195	0.199
20	—	0.746	0.854

Figures represent mean optical density obtained from two observations.

b) Effect of storage of salicylaldehyde reagent on color development : On standing at room tempereture, the ability of salicylaldehyde reagent to form colored substance was improved, to some extent, in three days as shown in Fig. 5. (This period could not be shortened by incubating the reagent at 37°). Little reduction in activity of the reagent occurred for the following two weeks.

5. Specificity

Under the described conditions, the following substances at the concentration found in normal blood neither gave any significant degree of color development with salicylaldehyde nor did they interfere the reaction for acetone : pyruvic acid (10 γ), lactic acid (10γ), lactic acid (50 γ), glucose (100 γ—see also Table V).

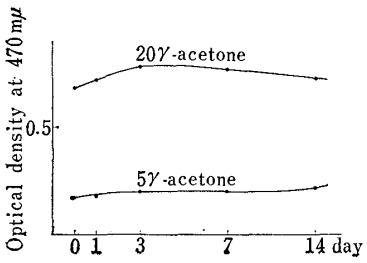

Fig. 5. Effect of Storage of Salicylaldehyde Reagent on Color Development
One per cent salicylaldehyde solution stored at room temperature was used.

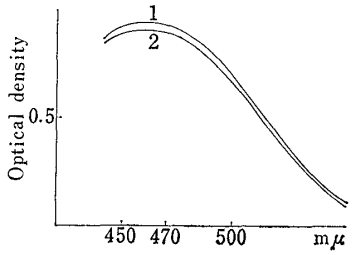

Fig. 6. Absorption Spectrum
1 : Without oxidizing reagent (20 γ acetone) : diluted with H_2O to give a total volume of 5.5 ml. before reading.
2 : With oxidizing reagent (50 γ acetone) : to 3.0 ml. of the reaction mixture, the same volume of H_2O was added before reading.

6. Absorption Spectrum

The colored product showed the maximum absorption near 470 mμ in the presence or absence of the oxidizing reagent (Fig. 6).

7. Recovery of 3-Hydroxybutyric Acid from Serum

Table IV shows the recovery test of 3-hydroxybutyrate added to various amounts

of serum ranging from 0.04 to 0.20 ml. The recovery was calculated on the basis of optical density found/optical density expected times 100. When fasted rat serum up to 0.10 ml. was used (Table IV—I), the recovery was on average nearly 100%, while over 0.20 ml. of serum, only 81.7% of added 3-hydroxybutyrate was recovered. As it was tentatively supposed that this low recovery was due to the relatively high final concentration of 3-hydroxybutyrate compared with the case of 0.05 or 0.10 ml. of serum, glu-

Table IV. Recovery of 3-Hydroxybutyrate from Serum

Serum concn. (ml./ml.)	B. H. B.[a] added (γ)	I			II		
		O. D.[b] expected	O. D. found	% of recovery	O. D. expected	O. D. found	% of recovery
0.04	5				0.131	0.105	84.6
	10				0.219	0.181	81.8
	15				0.305	0.268	87.1
	20				0.394	0.364	92.3
0.05	5	0.236	0.221	91.6	0.116	0.101	83.3
	10	0.327	0.334	100.0	0.178	0.167	94.5
	15	0.398	0.421	105.0			
	20				0.308	0.320	103.1
0.10	5	0.395	0.379	95.0	0.162	0.132	81.2
	10	0.486	0.507	104.1	0.228	0.169	73.9
	15	0.557	0.638	114.2			
	20				0.354	0.265	77.1
0.20	5	0.638	0.525	82.8	0.201	0.148	75.0
	10	0.729	0.592	80.9	0.263	0.158	61.6
	15	0.800	0.645	81.3	0.349	0.189	54.3
	20				0.393	0.225	59.0

a) 3-Hydroxybutyric acic butyric
b) Optical density (Each value presents the mean of two observations.)
I : Fasted rat serum
II : Glucose-fed rat serum

cose-fed rat serum which contained much smaller amount of ketone bodies (see also Fig. 8) was used in the subsequent experiment. However, as shown in Table IV—II, a still lower recovery was obtained than in the former case. The low recovery found in fed rat serum is not ascribed to the higher concentration of glucose in the serum sample because glucose, when added to the serum, neither influenced the recovery of 3-hydroxybutyric acid nor caused color formation with salicylaldehyde, in the range from 100 to 400 mg. % (Table V). These facts might suggest that the poor recovery found in serum glucose-fed rats was due to any factors other than glucose.

Table V. Effect of added Glucose on the Recovery of 3-Hydroxybutyric Acid

Serum (0.1 ml.)	Glucose added (γ)	O. D.[a]	Serum (0.1 ml.)	Glucose added (γ)	O. D.[a]
—	100	0	+	100	0.224
—	200	0	+	200	0.238
—	400	0	+	400	0.217
—	1000	0.006	+	1000	0.193
+	—	0.225			

a) Means of two observations

8. Standard Curves

Standard curves of acetone, acetoacetate and 3-hydroxybutyrate are shown in Fig. 7. The linear relationship between the optical density and the concentration of acetone, was obtained in a range of 1 to 20γ, and for acetoacetic and 3-hydroxybutyric acids, in a range of 5 to 40 γ.

9. Standard Procedure

According to the experimental observations obtained above, the following standard

procedure was established.

One-tenth ml. of whole blood (obtained from a small incision at the tip of tail of the rat) is delivered into 1.20 ml of distilled water in a small centrifuge tube and after hemolysis by shaking, 0.20 ml. of 12% perchloric acid in slowly added under continuous agitation. The precipitation formed is centrifuged off. One ml. of the supernatant is then put in the glass-stoppered pyrex tube (12×120 mm.) and 0.5 ml. of oxidizing reagent (0.2% $K_2Cr_2O_7$ in 6*N* H_2SO_4) is added. After heating in a boiling water bath at 100° for 1 hours 2.0 ml. of a mixture of 5% sodium sulfite, 6*N* potassium hydroxide and 2% salicylaldehyde (1:1:2) and 2.5 ml. of 50% potassium hydroxide is added to all tubes, following which they are kept at 0° for 16 hr. The color formed is measured at 470 ml. against a blank which has been prepared by using distilled water instead of the blood sample to be analyzed, and is set at 100% transmission. When 50% potassium hydroxide is added, precipitate occurs which does not interfere with the subsequent analytical procedure but is preferably removed by slight centrifugation before reading.

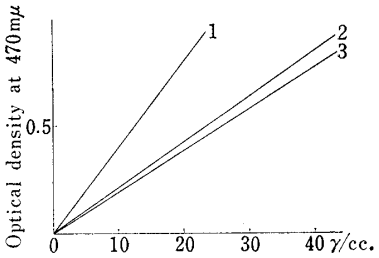

Fig. 7. Standard Curves
1 : acetone
2 : acetoacetic acid
3 : 3-hydroxybutyric acid

The amount of the ketone bodies is calculated as acetone from the equation. in which E is optical density, F the standardization factor, V the volume of the blood sample in the reaction solution, and 0.56 the conversion factor of 3-hydroxybutyric acid

$$\left[\mathrm{Me_2CO\ mg.\ \%} = \frac{E \times F \times 0.56 \times 100}{V \times 1000} \right.$$

into acetone (=moles of Me_2CO÷moles of 3-hydroxybutyrate). The F is made from 3-hydroxybutyrid acid standard curve.

10. Practical Application of the Method (Effect of Glucose on the Ketone Levels in Blood)

The standard method was applied to the experiment in which the effect of glucose feeding to fasted rats on blood ketone body levels was followed for 150 minutes. on 0.1 ml. blood specimen optained from tip of the tail (Fig. 8). As illustrated in Fig. 8, blood ketone body level of the control animal was maintained within reasonably small variations and oral administration of glucose brought about a marked hypoketonemia which was in fair agreement with the findings by other investigators.

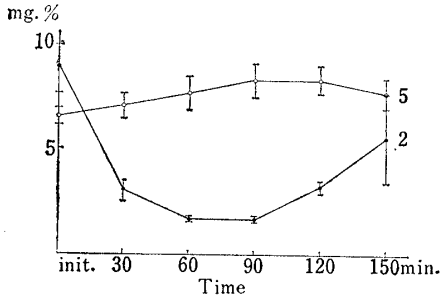

Fig. 8. Effect of Oral Administration of Glucose on Blood Ketone Body Levels
Dose of glucose : 5g./kg. body weight (in 50% solution)
•—• : Treated
○—○ : Control mg. %, as acetone
Figures in parentheses present number of examples.

Discussion

As pointed out in previous publications,[12,13] there has been recognized the wide scatter of values for human blood ketone bodies which often minimized the metabolic response to be observed. Such difficulties may be overcome by the use of well-controlled experimental animals as blood donors which are expected to have relatively constant response to metabolic stimuli. Many of the analytical methods so far proposed, however, suffer from a lack of both sensitivity and accuracy when applied to the assay of minute volume of blood samples obtainable at intervals from small experimental animals.

In the reported procedure, technical simplicity and accuracy gained by carrying out the whole process as an one-unit operation in a glass-stoppered pyrex tube without any transfer until colorimetry, together with higher sensitivity, have made it possible to observe the periodical change of rat blood ketone bodies as exemplified in Fig. 8.

Greenberg and Lester[5] carried out the conversion of the keto acids to acetone in a single step using acid dichromate, but Michaels, *et al.*[8] found that heating in acid dichromate caused unavoidable losses of acetoacetate and low recovery of 3-hydroxybutyrate and hence they introduced the two-step heating method, which was supported by Werk, *et al.*[7] Though more acetoacetate was recovered in two-step procedure conducted in this study, confirming the previous findings, the simpler one-step method was involved in routine assay technique; because practically identical values were found in blood ketone body levels determined by either of the two different methods (Table II). The two-step method will be preferable if more precise data should be required for each component of ketone bodies.

The time required for the development of color, if desired, can be shortened by heating as in the methods of Krog and Lund[14] or Cantoni,[15] or by raising potassium hydroxide concentration in the reaction mixture. The rise in either temperature or alkali concentration on color formation, however, will result in a marked reduction of sensitivity as evident in Figs. 1 and 2. Furthermore, this lowered sensitivity could not be compensated for by taking larger amount of blood samples owing to depressed recovery of 3-hydroxybutyric acid in such a case (Table IV-I, II).

Perchloric acid employed in the present technique as deproteinizing reagent may not be replaced by trichloroacetic acid, because the filtrate prepared by adding the latter tends to form a small froth during the course of color development.

In conclusion, owing to its high sensitivity and technical simplicity, the method reported here may safely be employed in the experimental studies, where the repeated withdrawal of specimen is required to follow the change of blood ketone bodies of small animals.

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Summary

A salicylaldehyde method for microdetermination of blood ketone bodies was extensively studied. A wide variety of factors influencing the oxidation of keto acids to acetone and color development was examined, and a simple and sensitive method was standardized by which periodical analysis of ketone bodies on 0.1 ml. of rat whole blood can be routinely performed.

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12) T.E. Weichselbaum, M. Somogyi : J. Biol. Chem., **140**, 5 (1941).
13) E.E. Werk, H.C. Knowles : Diabetes, **10**, 22 (1961).
15) O. Cantoni : Biochem. Z., **274**, 45 (1934).