Separation of isomers

The separation of the *n*- and *iso*-C_4, C_5 and C_6 ions was incomplete in 20 hr. By running the chromatogram for approx. 60 hr., complete separation of the C_4 and C_5 isomers could be achieved. The high R_F values of the *n*- and *iso*-C_6 ions would necessitate the use of excessive lengths of paper to separate these isomers completely on a one-dimensional chromatogram.

SUMMARY

1. The separation of the lower fatty acids as anions by paper chromatography is described using for development mixtures of alcohols with aqueous ammonia. Separation of the *n*- and *iso*-C_4 and C_5 anions has also been achieved.

2. The anions and cations from quantities of salts as low as 5 μg. can be detected on the developed chromatograms.

REFERENCES

Brown, F., Hirst, E. L., Hough, L., Jones, J. K. N. & Wadman, W. H. (1948). *Nature, Lond.*, **161**, 720.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Elsden, S. R. (1946). *Biochem. J.* **40**, 252.
Elsden, S. R. (1950). *Biochem. Soc. Symp.* **3**, 74.
Fink, K. & Fink, R. M. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 654.
Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1948). *J. chem. Soc.* p. 1679.
Goodall, R. R. & Levi, A. A. (1946). *Nature, Lond.*, **158**, 675.
Hirst, E. L., Hough, L. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 928.
Lederer, M. (1948). *Nature, Lond.*, **162**, 776.
Lester Smith, E. (1942). *Biochem. J.* **36**, xxii.
Linstead, R. P. (1949). *Nature, Lond.*, **163**, 598.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
Moyle, V., Baldwin, E. & Scarisbrick, R. (1948). *Biochem. J.* **43**, 308.
Peterson, M. H. & Johnson, M. J. (1948). *J. biol. Chem.* **174**, 775.
Ramsey, L. L. (1948). *J. Ass. off. agric. Chem., Wash.*, **31**, 164.
Ramsey, L. L. & Patterson, W. I. (1945). *J. Ass. off. agric. Chem., Wash.*, **28**, 644.
Ramsey, L. L. & Patterson, W. I. (1948). *J. Ass. off. agric. Chem., Wash.*, **31**, 139.
Smith, H. W. & White, T. A. (1929). *J. phys. Chem.* **33**, 1953.

Cholesterol Metabolism

2. CHOLESTEROL METABOLISM IN THE RAT

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The experiments to be described arose from an observation by Cook (1937) that when cholesterol in a fat-containing diet was fed to rats there was a loss of the sterol. Cook (1938) constructed a lipid and sterol balance sheet and showed that there was an apparent correlation between the amount of cholesterol metabolized and the increase of fatty acids excreted in the faeces. An acid was isolated from the faecal lipids, but it could not be stated definitely that it was formed from the breakdown of cholesterol. Polgar (1948) investigated the fatty acids present in the tubercle bacillus separating them by the fractional crystallization of their acetol esters ($R.CO_2.CH_2.CO.CH_3$) and treating the latter with reagents for ketones such as semicarbazide or 2:4-dinitrophenylsemicarbazide. It was decided to apply these methods to the study of the acids produced in the metabolism of cholesterol. Different types of fat were administered with the cholesterol to ascertain if these influenced its metabolism.

There is little exact information on the changes which cholesterol undergoes in the animal body and this work is part of a larger study of the metabolism of this compound in various animal species.

METHODS

Animals. Piebald male rats about 6 months old and of average weight 300 g. obtained from the Rowett Institute, Aberdeen, were used in all experiments. The animals were housed in a room maintained at a temperature of 22° ± 1° in metabolism cages fitted with Hopkins's separators, the urine and faeces being collected separately. The food was weighed daily, moistened and placed in containers, any food spilled being returned to the dish. The animals were allowed free access to water.

Table 1. *Rat diets*

(The table gives the weights (in g.) of constituents in the 'control' diets. The 'cholesterol' diet for each experiment was identical with the 'control' diet except for the addition of 20 g. cholesterol (25 g. in Exp. II).)

Exp. no. ...	I	II	III, IV	V	VI, VIII	VII
Constituent	(cooking fat)	(butter fat)	(olive oil)	(low fat)	(olive oil + rat cake)	(rat cake)
Caseinogen (1)	230	230	230	230	—	—
Maize starch	400	400	400	442	—	—
Cane sugar	170	170	170	176	—	—
Fat (2)	200	200	200	—	200	—
Salt mixture (3)	50	50	50	53	—	—
Dried yeast (4)	75	75	75	80	—	—
Cod liver oil (5)	30	30	30	—	—	—
Radiostol (6)	—	—	—	{1 drop/	—	{1 drop/
Avoleum (7)	—	—	—	rat/day}	—	rat/day}
Rat cake (8)	—	—	—	—	1000	1000

(1) Unextracted caseinogen supplied by Glaxo Laboratories Ltd. was used.
(2) The cooking fat, butter fat and olive oil were commercial samples obtained locally.
(3) The salt mixture of McCollum & Davis (1915) was used.
(4) Dried yeast—The Distillers Co. Ltd.
(5) 'Seven Seas' Cod liver oil—British Cod Liver Oils (Hull and Grimsby) Ltd.
(6) Radiostol—Potency 3000 i.u. Vitamin D/g. British Drug Houses Ltd.
(7) Avoleum—Potency 30,000 i.u. Vitamin A/g. British Drug Houses Ltd.
(8) Rat cake supplied by North-Eastern Agriculture Co-operative Society Ltd., Aberdeen. This contains 5% ether-soluble lipid and 12 mg. sterol/100 g

Diets. The diets, details of which are given in Table 1, are of two types, viz. 'Synthetic' (used in early experiments) which were made up with known compounds, and 'Rat cake', having as its basis a mixture of foodstuffs. The synthetic diet offered no advantages over the rat-cake diet which is convenient and readily taken by the animals. The bulk of the faeces formed on this diet was much greater than with the synthetic diet.

Cholesterol. Recrystallized cholesterol (Glaxo Laboratories Ltd.) was used. The components of the diets were well mixed and in those diets containing fat and cholesterol the latter was dissolved in the warm oil before being mixed with the other ingredients.

Extraction of faeces. The faeces were collected daily and dried *in vacuo* at 95° to constant weight. They were then extracted continuously with ether, first in the pellet form and then after being powdered, for a total of 30 hr. In Exps. VI–VIII the ether-extracted faeces were further extracted with 3 vol. of boiling ethanol to remove salts of fatty acids and other materials insoluble in ether. The ether or ethanol was removed by distillation and the extracted material weighed. This was then saponified for 6 hr. with 10% (w/v) ethanolic KOH solution, 6 ml./g. lipid (Hilditch, 1940). Water (3 vol.) was then added and the solution was extracted with an equal volume of ether divided into three portions. The unsaponifiable matter (U.M.) was recovered from the water-washed ether and weighed. The soap solution was acidified with 10% (v/v) H_2SO_4 and extracted with 0·5 vol. ether divided into two portions. The ether-soluble acids were weighed. This procedure does not remove all the volatile acids, but these form a small percentage of the total acids. The relation of the production of volatile acids to cholesterol metabolism will be reported later. In Exps. VI–VIII the acidic fraction was separated into light petroleum (b.p. 40–60°) soluble and insoluble fractions by the following procedure. Light petroleum (100 ml./g. total acids) was added to the flask, the mixture well shaken for 20 min., allowed to stand for 24 hr. and then centrifuged. The soluble material was recovered and weighed. The insoluble material was dissolved in ethanol, recovered and weighed.

Free and total sterols. These were determined on samples of the unsaponified lipids by the method of Schoenheimer & Sperry (1934). In this communication the values for total sterol alone are reported.

Livers. At the end of an experiment the animals were bled under light anaesthesia, the livers removed and weighed. They were then coarsely chopped, dried *in vacuo* at 100° and when dry were extracted with ethanol-ether (3:1, v/v). The fractionation of the lipid was as for the faeces.

Urine. In some experiments this was extracted after acidification with 1 vol. of ether divided into three portions.

RESULTS

The duration of experiments, weights of rats, total food, fat and cholesterol ingested are shown in Table 2. The inclusion of cholesterol in the diet did not affect the appetites of the animals and their body weight increased during the period of feeding.

The faecal excretion of lipid (ether-soluble, and ethanol-soluble, where determined), unsaponifiable matter (U.M.), sterol, total acids and light petroleum-insoluble acids (where determined), are shown in Table 3.

The discrepancy between the sum of the values for U.M. and total acids and the value for total lipid is explained by the facts that some lipid was present as glyceride and also that some volatile acids are not completely extracted by ether and when extracted are lost in the drying process.

The urines were not investigated systematically, but in Exp. VII the control animals excreted 4·6 g.

Table 2. *Duration of experiment, weight of rats, total food, fat and added cholesterol ingested*

(C, control group. S, group fed added cholesterol.)

Exp. no.	Fat	Group	No. of animals	Increase or decrease in wt. during exp. (g.)	Duration of experiment (days)	Total food (g.)	Fat (g.)	Added cholesterol (g.)
I	Cooking fat	C	4	−193	17	1077	186	0
		S	4	+115	18	1111	189	18·9
II	Butter fat	C	2	+135	34	1090	189	0
		S	2	+120	28	1065	181	22·6
III	Olive oil	C	2	+31	15	532	92	0
		S	2	+205	22	1093	186	18·6
IV	Olive oil	C	2	+13	45	1102	191	0
		S	4	+362	35	2242	382	38·2
V	Low fat	C	2	+90	22	950	0	0
		S	2	+105	22	970	0	19·0
VI	Olive oil + rat cake	C	2	+46	39	1125	188	0
		S	2	+47	31	1150	189	18·9
VII	Rat cake	C	6	+442	53	6900	0	0
		S	6	+356	53	7000	0	137·0
VIII	Olive oil + rat cake	C	2	+70	30	1083	180	0
		S	2	+21	30	1349	221	22·1

Table 3. *Faecal lipids*

(All wts. in g.; —, means not measured.)

Exp. no.	Group	Wt. dry faeces	Lipid: Ether-soluble (*A*)	Lipid: Ethanol-soluble (*B*)	Unsaponifiable matter (U.M.)	Total sterol	Total acids in: *A*	Total acids in: *B*	Light petroleum-insoluble acids in: *A*	Light petroleum-insoluble acids in: *B*
I	C	62·3	6·7	—	3·0	—	3·3	—	0·2	—
	S	81·6	24·6	—	16·6	—	6·8	—	0·5	—
II	C	59·5	5·9	—	1·9	1·1	3·8	—	0·3	—
	S	82·6	30·9	—	20·4	—	7·7	—	0·4	—
III	C	71·0	3·2	—	1·0	—	1·7	—	0·1	—
	S	69·2	26·0	—	13·7	11·1	6·8	—	0·8	—
IV	C	56·0	5·8	—	2·3	1·2	2·9	—	0·1	—
	S	479·3	44·0	—	29·1	—	11·4	—	1·6	—
V	C	49·0	3·4	—	1·1	0·8	1·7	—	0·1	—
	S	71·0	19·1	—	16·7	12·6	2·3	—	0·4	—
VI	C	211·0	10·6	10·4	2·0	1·0	6·5	4·2	0·6	1·0
	S	285·0	29·2	7·1	15·9	10·7	12·1	6·2	2·1	2·1
VII	C	1886	82·2	30·4	15·7	13·6	56·3	16·3	2·2	4·3
	S	1900	221·7	40·7	121·6	106·4	76·5	17·6	5·3	7·8
VIII	C	240	20·5	3·4	3·7	—	12·8	1·7	1·1	0·6
	S	286	47·6	6·9	20·5	—	21·9	4·4	2·1	1·5

lipid and 19 mg. sterol and the cholesterol-fed animals excreted 3·8 g. lipid and 72 mg. sterol over the period of investigation. It would appear that little cholesterol is excreted via the kidneys.

The composition of the lipid components of the livers is given in Table 4. In Exps. II–IV, VI and VIII the animals receiving the cholesterol-containing diet all had 'fatty' livers. The animals on the low fat diets (Exps. V and VII) all showed normal livers. There is an increase in the amount of light petroleum-insoluble acids in the livers of the animals fed a high-fat cholesterol-containing diet (cf. Exps. VII and VIII). Cook (1937) had shown that in the rat little cholesterol is deposited in tissues other than the liver. Such material and the small quantity of urinary sterol are neglected in constructing a balance sheet of the U.M. metabolism, which is shown in Table 5.

It is convenient to discuss the balance of U.M. rather than of sterol, as the values for U.M. have been obtained gravimetrically. This communication is not primarily concerned with the conversion of cholesterol to non-acidic products which remain in the unsaponifiable fraction. The relation of the sterol to the unsaponifiable matter in faeces and livers is shown in Tables 3 and 4.

Table 4. *Liver lipids*

(—, means not measured.)

Exp.	Fat	Group	Livers as % body wt.	Lipid as % liver wt.	U.M. (g.)	U.M. As % lipid	Total sterol as % lipid	Total acids as % lipid	Light petroleum-insoluble acids (g.)
I	Cooking fat	C	3·3	3·2	0·1	7·1	—	69·3	—
		S	4·0	3·9	0·4	17·7	—	60·0	—
II	Butter fat	C	3·1	5·2	0·1	8·0	—	55·0	0·10
		S	3·5	8·5	0·4	16·0	—	55·2	0·14
III	Olive oil	C	3·2	5·3	0·1	9·1	—	72·7	—
		S	3·9	17·8	2·2	39·8	13·8	36·4	0·64
IV	Olive oil	C	3·2	7·1	—	—	5·0	—	0·11
		S	4·3	15·4	2·0	32·3	28·0	44·2	0·20
V	Low fat	C	3·8	3·5	—	—	5·0	—	—
		S	3·9	4·6	—	—	3·6	—	—
VI	Olive oil + rat cake	C	2·8	7·8	—	—	6·6	—	—
		S	4·0	11·7	1·1	35·3	30·6	48·3	—
VII	Rat cake	C	3·8	5·1	0·2	4·6	3·7	52·2	0·08
		S	4·7	3·5	0·4	11·6	11·6	60·7	0·06
VIII	Olive oil + rat cake	C	2·9	7·9	0·1	7·0	—	48·8	0·02
		S	5·5	17·3	2·1	30·6	—	38·3	0·20

Table 5. *Balance sheet of the* U.M. *metabolism on the various diets*

(The difference between the unsaponifiable matter (U.M.) of the ether extractable lipid of the control and cholesterol-fed animals has been taken as a measure of unchanged cholesterol. All weights in g.)

Exp. no.	Difference in faecal U.M. *A*	Difference in liver U.M. *B*	*A* + *B*	Cholesterol ingested *C*	Difference *C* − (*A* + *B*)
I	13·6	0·3	13·9	18·9	5·0
II	18·5	0·3	18·8	22·6	3·8
III	11·7*	2·1	13·8	18·6	4·8
IV	24·5*	2·0	26·5	38·2	11·7
V	15·6	0	15·6	19·0	3·4
VI	13·9	1·0	14·9	18·9	4·0
VII	105·9	0·2	106·1	137·0	30·9
VIII	16·8	2·1	18·9	22·1	3·2

* In these experiments twice the control value given in Table 3 was subtracted because in Exp. III, C group were on the diet for less time than S group and in Exp. IV two animals were in C group as compared with four in S group.

Table 6. *Loss of* U.M. *and increase in faecal acids on the cholesterol-containing diets*

('Loss of U.M.' taken from Table 5.)

Exp. no.	Loss of U.M. (g.)	Increase in total faecal acids in: Ether-soluble lipid (g.)	Increase in total faecal acids in: Ethanol-soluble lipid (g.)	Increase in light petroleum-insoluble acids in: Ether-soluble lipid (g.)	Increase in light petroleum-insoluble acids in: Ethanol-soluble lipid (g.)	Light petroleum-insoluble acids as % of U.M. loss: Ether-soluble lipid	Light petroleum-insoluble acids as % of U.M. loss: Ethanol-soluble lipid	Light petroleum-insoluble acids as % of U.M. loss: Total
I	5·0	3·5	—	0·3	—	6	—	—
II	3·8	3·9	—	0·1	—	3	—	—
III	4·8	5·1	—	0·7	—	15	—	—
IV	11·7	8·5	—	1·5	—	13	—	—
V	3·4	0·6	—	0·3	—	9	—	—
VI	4·0	5·6	2·0	1·5	1·1	37	27	64
VII	30·9	20·2	1·3	3·1	3·5	10	11	21
VIII	3·2	9·1	2·7	1·0	0·9	31	28	59

There is a loss of U.M. in all experiments, even when a diet low in fat is fed (see Exps. V and VII). The excretion of acids and the loss of U.M. are compared in Table 6. The results of the early experiments are not complete, as it was not then realized that additional lipid, particularly of an acidic nature, could be removed by treatment with boiling ethanol. It was also not known that the cholesterol degrada-

tion products could be concentrated by virtue of their relative insolubility in light petroleum (see below). Particularly is this so with Exps. I and II where there was a considerable manipulative loss of these products. There is an excess of total acids excreted in the faeces of the cholesterol-fed animals, especially those fed additional fat. This excess excretion of fatty acids parallels the loss of U.M. There is a marked increase in the excretion of light petroleum-insoluble acids particularly with the animals fed additional fat. In Exps. VI and VIII the increases correspond to 64 and 59 %, respectively, of the U.M. loss. The amounts produced on the low fat diets (Exps. V and VII) are considerably less. It may be of significance that in these animals the liver appearance was normal and that with the high-fat cholesterol-containing diets more light petroleum-insoluble acids are present in the livers.

Separation of acidic components

During the initial stages of this investigation the acids were separated by conversion to the methyl esters followed by fractional distillation *in vacuo*, the ordinary fatty acids being removed. The non-volatile residue contained optically active acidic products presumably derived from cholesterol. The residue of the material obtained by preliminary treatment of the faecal acids with light petroleum is a dark-brown gum. Attempts to crystallize it from the usual organic solvents have been unsuccessful. The crude material has an acid equivalent of 386 and from it salts (sodium, potassium and barium) may be prepared and recrystallized, but even these salts on decomposition and extraction give resinous acidic products.

The mixed faecal acids of Exp. VI were separated as follows. The acids (10·3 g.) were converted into their methyl esters by refluxing with methanolic H_2SO_4 and the esters were distilled up to 180° at 0·8 mm. The optically inactive distillate consisted of the methyl esters of fatty acids. These will be the subject of another communication. The residue (2·3 g.) was treated with light petroleum (25 ml., b.p. 40–60°) which 1·3 g. were insoluble. Chromatography of this petroleum-insoluble material in benzene solution over precipitated silica (80–180 mesh) gave a viscous oil (0·3 g.) easily soluble in methanol and most organic solvents except light petroleum. This product had a specific rotation $[\alpha]_D^{20°} + 27°$ in $CHCl_3$ (*c*, 2·2). The oil was hydrolysed by refluxing it with ethanolic KOH solution and the free acid was converted into the K salt and thence into the acetol ester by the method of Polgar (1948). The latter on heating in ethanolic solution with 2:4-dinitrophenylsemicarbazide, followed by chromatography of the resulting product over precipitated silica, gave 0·03 g. of a yellow powder, probably not crystalline, m.p. 98–99°. (Found: C, 57·2; H, 6·8; N, 13·4. $C_{47}H_{64}O_{14}N_{10}$ requires C, 56·9; H, 6·5; N, 14·1 %.) The above formula is that of a bis-dinitrophenylsemicarbazone acetol ester derived from a dibasic acid of composition $C_{27}H_{46}O_4$. There are indications that a number of optically active acids are present and these will be the subject of further investigations.

DISCUSSION

Numerous balance experiments on cholesterol have been carried out in various animal species (for review see Cook, 1942). From these experiments the conclusion may be drawn that cholesterol can be synthesized, probably from acetic acid units (Little & Bloch, 1950), or if large amounts of sterol are fed then it is broken down. It is probable that there is a balance between synthesis and degradation. Apart from non-acidic compounds such as coprosterol (Rosenheim & Webster, 1941) no other definite end products of metabolism have been isolated. Bloch, Berg & Rittenberg (1943) have suggested that cholesterol is converted to cholic acid, but this work is still unsubstantiated. Turfitt (1948) has indicated that certain bacteria can convert cholesterol to acidic products in which ring A has been disrupted at C_3–C_4.

The results reported here show that there is a loss of U.M., and therefore of cholesterol, and an increase in faecal acids particularly in the light petroleum-insoluble fraction. From this fraction has been isolated an optically active compound the empirical formula of which suggests that it is derived from cholesterol. Other optically active acids are present in the fraction.

When fat is present in the diet there is better absorption of the cholesterol and there is evidence that similar acidic compounds are formed in quantity in the liver. The striking increase in the excretion of the ordinary fatty acids on the high-fat cholesterol-containing diet still remains to be explained.

SUMMARY

1. Rats have been fed diets containing 1·6 % cholesterol in basal diets containing 16·6 % added fat (cooking fat, butter or olive oil) and diets low in added fat.

2. The faecal lipids have been fractionated and on all diets fed there is an increase in excretion of acids, this being greatest with the fat-containing diets.

3. There is a loss of unsaponifiable matter and hence of cholesterol on constructing a balance sheet and this loss is of the same order as the increase in the light petroleum-insoluble faecal acids.

4. From the light petroleum-insoluble fraction of the faecal acids the derivative of an optically active acid of probable empirical formula $C_{27}H_{46}O_4$ has been isolated.

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REFERENCES

Bloch, K., Berg, B. N. & Rittenberg, D. (1943). *J. biol. Chem.* **149**, 511.
Cook, R. P. (1937). *Biochem. J.* **31**, 410.
Cook, R. P. (1938). *Biochem. J.* **32**, 1191.
Cook, R. P. (1942). *Nutr. Abstr. Rev.* **12**, 1.
Hilditch, T. P. (1940). *The Chemical Constitution of Natural Fats*, 1st ed. London: Chapman and Hall.
Little, H. N. & Bloch, K. (1950). *J. biol. Chem.* **183**, 33.
McCollum, E. V. & Davis, M. (1915). *J. biol. Chem.* **20**, 641.
Polgar, N. (1948). *Biochem. J.* **42**, 206.
Rosenheim, O. & Webster, T. A. (1941). *Biochem. J.* **35**, 920.
Schoenheimer, R. & Sperry, W. M. (1934). *J. biol. Chem.* **106**, 745.
Turfitt, G. E. (1948). *Biochem. J.* **42**, 376.

Manometric Determination of L-Aspartic Acid and L-Asparagine

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Gale (1945) has developed specific and accurate methods employing bacterial decarboxylases by which six amino-acids—glutamic acid, ornithine, arginine, lysine, histidine and tyrosine—can be determined in quantities from 0·05 mg. upwards. The method for the determination of L-aspartic acid described in this paper supplements Gale's methods. It is based on the combined use of aspartic-glutamic transaminase and of the glutamic and oxaloacetic decarboxylases. In a system containing these three enzymes, as well as L-aspartic acid and an excess of α-ketoglutarate, the following three reactions take place:

(1) L-Aspartic acid + α-ketoglutaric acid $\xrightleftharpoons{\text{aspartic-glutamic transaminase}}$ L-glutamic acid + oxaloacetic acid,

(2) Oxaloacetic acid $\xrightarrow{\text{oxaloacetic decarboxylase}}$ pyruvic acid + CO_2,

(3) L-Glutamic acid $\xrightarrow{\text{glutamic decarboxylase}}$ γ-aminobutyric acid + CO_2.

The overall effect of these three reactions is:

(4) L-Aspartic acid + α-ketoglutaric acid = γ-aminobutryic acid + pyruvic acid + $2CO_2$.

As the products of reaction 1 are removed quantitatively by the reactions 2 and 3 the reversible reaction 1 proceeds quantitatively from left to right and two molecules of carbon dioxide are formed for each molecule of aspartic acid present. This principle can also be used for the determination of α-ketoglutaric acid. If an excess of aspartic acid is added to the system containing the three enzymes and an unknown quantity of α-ketoglutaric acid, the latter limits the extent of the reactions 1, 2 and 3, and two molecules of carbon dioxide are formed for each molecule of α-ketoglutaric acid.

L-Asparagine can be determined by this principle if the enzymes catalysing 1, 2 and 3 are supplemented by asparaginase which hydrolyses L-asparagine:

$$\text{Asparagine} = \text{aspartic acid} + NH_3.$$

If glutamic decarboxylase is omitted from the above series of catalysts, one molecule of carbon dioxide is formed for each molecule of aspartic acid or α-ketoglutaric acid, the balance of reactions 1 and 2 being:

L-Aspartic acid + α-ketoglutaric acid = glutamic acid + pyruvic acid + CO_2.

Thus a combination of transaminase, oxaloacetic decarboxylase and asparaginase may also be used for the manometric determination of aspartic acid, asparagine or α-ketoglutaric acid. The omission of glutamic decarboxylase halves the sensitivity of the method, but this simplified procedure may be more convenient when facilities for preparing glutamic decarboxylase are limited. The only special reagent required for the simplified procedure is a transaminase preparation which is easily obtainable.

METHODS

The first part of the paper is concerned with the study of the four catalysts required for the methods. *Clostridium welchii*, strain SR 12, served as a source of glutamic decarboxylase, heart muscle as a source of aspartic-glutamic transaminase, aniline as a catalyst for the decarboxylation of oxaloacetate and guinea pig serum or liver as a source of asparaginase.

Preparation of suspensions of Clostridium welchii

The medium used for sub-culture and for preparing inocula is essentially the 'Cooked Meat Medium' described by Lepper & Martin (1929). The medium for growing bulk supplies