SUMMARY

1. Several DNP-amino-acids have been prepared and characterized.

2. Several modifications of the method of measuring the free amino groups of proteins with 1:2:4-fluorodinitrobenzene are described.

3. The method has been applied to several haemoglobins. Horse and donkey haemoglobins contain six terminal valyl residues/molecule, adult human haemoglobin five terminal valyl residues, and cow, sheep and goat haemoglobins two terminal valyl and two terminal methionyl residues. If a molecular weight of 66,000 is assumed, human foetal haemoglobin contains 2·6 terminal valyl residues. Horse myoglobin has one terminal glycyl residue/molecule of molecular weight 17,000. The ϵ-amino groups of the lysine residues are free in the above proteins.

4. The significance of these results in relation to the structure of the haemoglobins and the correlation between structure and immunological specificity of proteins is discussed.

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Metabolism of Acetoacetate in Animal Tissues. 2

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The first paper on this subject (Krebs & Eggleston, 1945) was concerned with the anaerobic reactions of acetoacetate. It was shown that in isolated sheep heart two rapid reactions of acetoacetate occur:

acetoacetate + L-malate
= L-β-hydroxybutyrate + oxaloacetate,
acetoacetate + α-ketoglutarate
= L-β-hydroxybutyrate + succinate + CO_2.

These two reactions account for the *anaerobic* metabolism of acetoacetate in sheep heart muscle: all the acetoacetate removed was recovered, within the limits of error, as β-hydroxybutyrate. The present paper deals with the *aerobic* reactions of acetoacetate. Whilst this work was in progress some of its problems were solved by Buchanan, Sakami, Gurin & Wilson (1945) with the help of isotopic carbon. These workers showed beyond doubt that the carbon

skeleton of acetoacetate enters the tricarboxylic cycle.

The main contributions of the present paper are measurements of the rate of the oxidation of acetoacetate in various tissues and the effect on these rates of the physiologically occurring dicarboxylic acids, as well as quantitative estimations of the products of acetoacetate metabolism. The results are in agreement with the conception that acetoacetate is oxidized through the tricarboxylic cycle.

METHODS

Tissue preparations

Suspensions of minced tissue were used in the same way as in the previous experiments (Krebs & Eggleston, 1945), unless otherwise stated. The metabolic activities were interrupted after a specified period of incubation by addition of 0·25 vol. of 2N-HCl.

Analytical procedures

Changes in the previous methods concerned the following points.

Determination of pyruvate in the presence of oxaloacetate. Oxaloacetate was not found in significant quantities when heart or kidney were incubated with this substrate under anaerobic conditions (unless the period of incubation was very short). Aerobically, however, oxaloacetate was often present, presumably because it was continuously reformed from malate. The problem therefore arose of determining pyruvate in the presence of oxaloacetate. Carboxylase decarboxylates oxaloacetate under the conditions used for the determination of pyruvate, but the amounts of CO_2 split off are variable. Under the standard conditions for the determination of pyruvate the initial rate of CO_2 formation from oxaloacetate was about 15% of that from pyruvate. The rate of CO_2 production gradually slowed down and the reaction practically stopped after 6 hr. when about 1·2 moles CO_2 had been formed/mole oxaloacetate (Table 1). The slowing down of the rate was not due to inactivation of carboxylase, for addition of pyruvate showed that the enzyme was still highly active. In more concentrated yeast extracts (4 ml. yeast extract, 0·2 ml. 3M-acetate buffer, pH 5·0; 0·1 ml. 0·1M-substrate solution) the total yield of CO_2 from oxaloacetate was only 0·6 mole/mole. These observations indicate that there are other reactions of oxaloacetate besides decarboxylation, the extent of which varies with the conditions. Neuberg & Karczag (1911) found that their yeast suspension liberated 2 moles of CO_2 from 1 mole oxaloacetate.

Table 1. *Decarboxylation of oxaloacetate by yeast extract*

(Yeast extract from English brewer's yeast (see Krebs & Johnson, 1937). Conditions for the manometric determination of CO_2 evolution according to Krebs & Eggleston (1945). The main compartment of each cup contained 3 ml. water; 0·1 ml. 0·1M-substrate; 0·2 ml. 3M-acetate buffer, pH 5·0; side arm, 1 ml. carboxylase. The data given are corrected for a small CO_2 evolution from yeast without substrate.)

Time after carboxylase addition (min.)	Substrate: Pyruvate (224 μl.)	Substrate: Oxaloacetate (224 μl.)
	CO_2 evolved (μl.)	
2	84	11·5
4	160·5	25·5
8	217·5	61·5
12	224	89
20	224	136
40	224	211
120	227	251
180	—	259
240	—	269

The following procedure was proved satisfactory in overcoming the difficulties created by the irregular yields of CO_2 from oxaloacetate: A measured fraction of the acidified tissue suspension containing 0·4N-HCl was heated for 15 min. in a water bath at 100° and the pH was then adjusted to 5·0 by addition of 3M-sodium acetate. This treatment converts oxaloacetate quantitatively into pyruvate and CO_2 whilst not destroying preformed pyruvate. The determination of pyruvate in the solution thus yielded the sum of pyruvate and oxaloacetate. In another fraction of the tissue suspension oxaloacetate was determined by the aluminium sulphate method (Krebs & Eggleston, 1945). The difference represented the preformed pyruvate.

Determination of oxaloacetate in the presence of pyruvate and acetoacetate. The manometric method previously used was based on the observation that multivalent cations specifically decarboxylate β-keto-dicarboxylic acids (Krebs, 1942). In the course of the present work it was found that acetoacetate reacts also if pyruvate or other α-ketonic acids are present (Table 2). In other words, α-ketonic acids have a catalytic effect on the decomposition of acetoacetate by aluminium ions. It is very probable that this is due to the formation, by aldol condensation, of a dicarboxylic β-ketonic acid from the α-ketonic acid and acetoacetate, e.g.

$$\mathrm{COOH.CO.CH_3 + CH_3.CO.CH_2.COOH} \rightleftharpoons \mathrm{COOH.C(CH_3)(OH).CH_2.CO.CH_2.COOH.}$$

In general the effect of α-ketonic acids was not great because of the low concentration of these substances in the solutions to be tested. The magnitude of the effect was estimated by extrapolation. Warburg cups were filled as previously described for the determination of oxaloacetate, and readings were taken for a period of 60 min. after the addition of $Al_2(SO_4)_3$. Under the conditions of the test oxaloacetate was quantitatively decomposed after 40 min. The CO_2 evolution which occurred between 40 and 60 min. was taken as due to the decomposition of acetoacetate and twice this value was deducted from the 40 min. reading to obtain the amount of oxaloacetate in the solution. This correction factor was probably slightly too high as the rate of CO_2 evolution from mixtures of acetoacetate and α-ketonic acid slightly increased with time (Table 2).

Acetoacetate does not seem to form appreciable quantities of such a condensation product with a second molecule of acetoacetate as long as the concentration of acetoacetate is low (0·005M). At higher concentrations (0·25M final concentration) Al ions accelérate the decarboxylation: without Al^{+++}, 0·25% was decomposed in 60 min., and with Al^{+++} 2·1% (conditions as stated in Table 2).

Table 2. *Evolution of* CO_2 *from mixtures of acetoacetate with α-ketonic acids in the presence of aluminium salts*

(Conditions as given by Krebs & Eggleston (1945) for the determination of oxaloacetate. Each cup contained 0·2 ml. 0·1 M-acetoacetate in addition to the substrate stated below. Concentration of added substrate solutions 0·1 M.)

Time after Al^{+++} addition (min.)	Substrates added: 0	0·05 ml. pyruvate	0·2 ml. pyruvate	1·0 ml. pyruvate	0·05 ml. α-keto-glutarate	0·1 ml. α-keto-glutarate	0·2 ml. α-keto-glutarate
	CO_2 evolved (μl.)						
10	0	0	19	80	0	7	32
20	0	5	44	147	0	14	64
40	0	17	93	232	0	29	123
60	0	26	126	272	3·5	46·5	165
120	4	62	188	320	17	88·5	249
240	14	86	239	343	—	—	—

Table 3. *Substances simulating β-hydroxybutyrate in the method of Van Slyke* (1917) *and of Edson* (1935)

Substance	Amount added (mg.)	'β-Hydroxybutyrate' formed (mg.)	Yield (mg. β-hydroxybutyrate/100 mg. substance tested)
L-Lactic acid	18	6·31	35·1
	9	2·80	31·1
	4·5	0·99	22·0
	2·25	0·04	1·78
	1·125	0·04	3·56
L-Malic acid	26·8	2·03	7·6
	13·4	1·14	8·5
	6·7	0·21	3·1
Fumaric acid	23·2	4·65	20·0
	11·6	1·80	15·5
	5·8	0·48	8·3
α-Ketoglutaric acid	29·2	0·287	0·98
	14·6	0·093	0·61
	7·3	0·032	0·23
Pyruvic acid	17·6	0·505	2·87
Citric acid	38·4	0·884	2·3
Succinic acid	23·6	Nil	Nil

Determination of β-hydroxybutyrate. The method previously used (Edson, 1935; Krebs & Eggleston, 1945) was modified in the following points in order to increase accuracy. Instead of 1 ml. 5% potassium dichromate, 2·5 ml. were added. The precipitate formed after the dichromate oxidation was filtered off when the solution had cooled to room temperature. Sintered glass funnels (3 G 3) allowing about 5–7 ml. fluid to filter/min. were used. Under the conditions of the determination 1·0 ml. 0·01 N-iodine were equivalent to 0·166 mg. β-hydroxybutyric acid in the filtrate.

Several substances present in the solution were found to simulate small quantities of β-hydroxybutyrate. Van Slyke (1917, 1929) already found this for lactate provided it was present in fairly large quantities. Data on the errors caused by various substances are given in Table 3. The concentrations of interfering substances were known and the data in Table 3 could thus be used for correcting results. The correcting factor was generally below 5% of the amounts of β-hydroxybutyrate as the quantities of the interfering substances in the sample were small, viz. lactic acid between 0·3 and 1·2 mg.; malic acid between 0·5 and 2 mg.; fumaric acid between 0·5 and 1 mg.

The errors were of the same order in pure solutions and in mixtures with β-hydroxybutyrate. Attempts to increase the specificity of the method by separating β-hydroxybutyrate from the interfering substances through chromatography were unsuccessful.

Fumaric acid was in a number of cases determined by the manganometric method of Straub (1935).

Citric acid. Water was used instead of dioxan to dilute the final alkaline Na_2S solution to the required volume in the colorimetric determination. The colour was found to be more stable in this medium than in aqueous solutions of commercial 'pure' dioxan.

RESULTS

Comparative rates of reduction and oxidation of acetoacetate

In Table 4, experiments on sheep heart and sheep kidney cortex are recorded in which the amounts of acetoacetate removed and β-hydroxybutyrate

Table 4. *Amounts of acetoacetate removed and β-hydroxybutyrate formed*

(All data refer to 4 ml. suspension; acetoacetate 'oxidized' refers to the amounts removed which are not accounted for by the formation of β-hydroxybutyrate.)

Exp. no.	Tissue and medium	Acetoacetate added (μl.)	Other substrates added (μl.)	Period of incubation (min.)	Changes in N_2: Acetoacetate removed (μl.)	Changes in N_2: β-Hydroxybutyrate formed (μl.)	Changes in N_2: Acetoacetate 'oxidized' (μl.)	Changes in O_2: Acetoacetate removed (μl.)	Changes in O_2: β-Hydroxybutyrate formed (μl.)	Changes in O_2: Acetoacetate 'oxidized' (μl.)
1	Sheep heart, minced	1692	—	60	—	—	—	910	527	383
	1 part + 9 parts phosphate	1692	Oxaloacetate (224)	60	—	—	—	1110	646	464
	saline	1692	Oxaloacetate (112)	60	—	—	—	1032	580	452
2	Ditto	1760	—	60	377	331	46	1062	782	280
		1760	Fumarate (448)	60	1215	1218	~Nil	1245	1040	205
3	Ditto	846	—	40	—	—	—	435	406	29
		846	Fumarate (896)	40	—	—	—	656	620	36
		846	Oxaloacetate (896)	40	—	—	—	604	561	43
4	Ditto	850	—	20	—	—	—	310	304	6
		850	—	40	—	—	—	493	349	144
		850	—	120	—	—	—	492	184	308
		850	Oxaloacetate (896)	20	—	—	—	569	375	194
		850	Oxaloacetate (896)	40	—	—	—	764	409	355
		850	Oxaloacetate (896)	120	—	—	—	691	156	535
5	Ditto	878	—	40	—	—	—	558	325	233
		878	Oxaloacetate (896)	40	—	—	—	691	417	274
		878	Fumarate (896)	40	—	—	—	706	427	279
6	Ditto	1740	—	30	—	—	—	410	304	106
		1740	Oxaloacetate (896)	30	—	—	—	825	521	304
		1740	Fumarate (896)	30	—	—	—	759	573	186
7	Sheep kidney cortex minced	1856	—	40	386	366	20	653	227	426
	1 part + 9 parts phosphate	1856	Fumarate (896)	40	751	778	~Nil	656	309	347
	saline	1856	Oxaloacetate (896)	40	651	485	166	656	279	377
8	Ditto	1760	—	60	360	322	38	355	175	180
		1760	Fumarate (448)	60	680	658	22	550	320	230
9	Ditto	872	—	60	—	—	—	394	182	212
		872	Fumarate (896)	60	—	—	—	642	466	176
		872	Oxaloacetate (896)	60	—	—	—	604	366	238
10	Ditto	829	—	60	—	—	—	204	127	77
		829	Fumarate (896)	60	—	—	—	359	289	70
		829	Oxaloacetate (896)	60	—	—	—	341	158	183
11	Ditto	1285	—	90	—	—	—	647	229	418
		1285	Oxaloacetate (1344)	90	—	—	—	1084	509	575
12	Ditto	1647	—	30	—	—	—	220	161	59
		1647	Oxaloacetate (1792)	30	—	—	—	622	287	335
13	Ditto	847	Oxaloacetate (896)	20	—	—	—	261	181	80
		847	Oxaloacetate (896)	40	—	—	—	436	274	162
		847	Oxaloacetate (896)	80	—	—	—	613	239	374
		847	Oxaloacetate (896)	160	—	—	—	630	212	418
14	Ditto	1871	—	40	374	236	138	—	—	—
		1871	Oxaloacetate (1792)	40	661	436	225	—	—	—
15	Ditto	942	Oxaloacetate (448)	30	244	183	61	324	164	160
16	Sheep kidney cortex minced 1 part + 2·6 parts NaCl-$NaHCO_3$ (medium of Breusch, 1943 *b*)	888	Oxaloacetate (896)	20	588	421	167	583	433	150
17	Ditto	848	Oxaloacetate (1792)	30	673	501	172	—	—	—
		848	Oxaloacetate (1792) + pyruvate (820)	30	704	488	216	—	—	—
18	Ditto	1742	Oxaloacetate (3584)	30	1406	1081	325	—	—	—
		1742	Oxaloacetate (3584) + pyruvate (1650)	30	1406	1046	360	—	—	—
19	Sheep kidney cortex minced	890	Oxaloacetate (1792)	15	357	283	74	—	—	—
	1 part + 7 parts phosphate-$NaHCO_3$ saline	890	Oxaloacetate (1792)	30	490	438	52	—	—	—
		890	Oxaloacetate (1792)	60	603	620	Nil	—	—	—

formed are compared. The data indicate the following:

(1) Anaerobically, as in the previous experiments (Krebs & Eggleston, 1945), almost all acetoacetate removed in sheep heart was accounted for by the formation of β-hydroxybutyrate. A small excess was sometimes found when no additional substrate was added, but this was probably due to the non-enzymic ketone decomposition. No correction was applied for ketone decomposition, and as the average acetoacetate concentration during the experiment was usually higher when no further substrate was added (because the reduction was slower), the error in the calculation of the amounts 'oxidized' arising from ketone decomposition was larger in the absence of additional substrates. In sheep kidney the quantities of acetoacetate 'oxidized' anaerobically exceeded the experimental error if oxaloacetate was present (Exp. nos. 7, 14, 16, 17, 18).

(2) Aerobically a large proportion of the acetoacetate removed was still due to reduction to β-hydroxybutyrate, especially in short experiments in accordance with results published by Quastel & Wheatley (1935). On prolonged incubation, however, the amount of 'oxidized' acetoacetate gradually increased, whilst the amounts of β-hydroxybutyrate, after a rapid increase during the early periods of incubation, showed a fall in the later periods. It thus appears that in the early stages, when the acetoacetate concentration is high, the system acetoacetate ⇌ β-hydroxybutyrate reacts predominantly from left to right. Later, when most of the added acetoacetate has disappeared the reverse reaction predominates (Exps. 4 and 13).

(3) The amounts of acetoacetate oxidized aerobically were increased in many experiments by oxaloacetate or fumarate, both in heart muscle and in kidney. This may be taken as supporting the view that the four-carbon dicarboxylic acids play some part in the oxidation of acetoacetate (Quastel & Wheatley, 1935; Edson & Leloir, 1936; Breusch, 1943*a*, *b*; Wieland & Rosenthal, 1943; Lehninger, 1946), a conclusion which is not invalidated by the fact that the effects did not occur in every experiment (since the tissues are known to contain considerable quantities of precursors of oxaloacetate).

(4) In heart muscle, the initial, that is maximum, rate of aerobic reduction of acetoacetate calculated in terms of $Q_{\beta\text{-hydroxybutyrate}}$, assuming the dry weight to be 1/5 of the wet weight, was 11·4 without oxaloacetate, 14·1 with oxaloacetate (Table 4, Exp. 3). The maximum rates of oxidation without oxaloacetate (same exp., period 20–40 min.) in heart were 5·2 and 7·3 with oxaloacetate. In this tissue the maximum rates of reduction were thus about twice those of oxidation. In kidney cortex the corresponding rates of reduction were 4·0 without and 7·2 with oxaloacetate, the rates of oxidation 1·5 without and 8·4 with oxaloacetate (Table 4, Exp. 12). In kidney the maximum rates of oxidation and reduction in the presence of oxaloacetate therefore were of the same order.

Products of acetoacetate removal

Examples giving comprehensive analyses of the products of acetoacetate metabolism are shown in Table 5. The comparison of the yields of the various substances on the addition of oxaloacetate, with those found on addition of both oxaloacetate and acetoacetate, shows that acetoacetate caused an increase in the yields of citrate and of succinate,

Table 5. *Acetoacetate and oxaloacetate in sheep kidney*

(48 ml. suspension containing 1 part minced kidney cortex and 9 parts phosphate saline pH 7·4. All quantities are in μl. and refer to 48 ml. suspension. The three experiments were carried out on different kidneys.)

	Exp. 1 N_2 (40 min. incubation)			Exp. 2 O_2 (30 min. incubation)			Exp. 3 O_2 (30 min. incubation)	
Substrates added:								
Acetoacetate	22,460	—	22,460	19,760	—	19,760	—	12,140
Oxaloacetate	—	21,500	21,500	—	21,500	21,500	21,500	21,500
Substances found after incubation:								
Acetoacetate	17,970	~0	14,520	17,360	—	12,300	~0	6,230
β-Hydroxybutyrate	2,830	~0	5,210	1,670	—	3,410	~0	2,915
Oxaloacetate	~0	1,210	1,850	—	2,670	4,400	1,640	2,770
Pyruvate	~0	4,070	4,750	—	—	—	550	1,400
Succinate	~0	1,270	2,005	—	320	1,360	240	342
α-Keto-(plus α-hydroxy)-glutarate	1,110	1,605	1,655	1,275	2,160	550	725	~0
Fumarate	—	2,765	880	—	—	—	—	—
L-Malate	~0	4,605	3,155	—	3,640	3,240	2,590	2,500
Citrate	10	156	1,690	—	1,530	3,240	1,740	3,820
Calculated changes:								
Acetoacetate reduced	2,830	—	5,210	1,670	—	3,410	—	2,915
Acetoacetate 'oxidized'	1,660	—	2,730	730	—	4,050	—	2,995

whilst the yields of α-keto(plus α-hydroxy)glutarate were decreased. On balance, less oxaloacetate disappeared when acetoacetate was present. The effects of oxygen on the yields were altogether not very great, the most notable difference being the higher yield of citrate in O_2.

The observations on the differences in O_2 and N_2 are in general in accordance with the view that aerobically the same reactions occur as anaerobically with certain additional reactions, namely, some of the oxidative steps of the tricarboxylic cycle and the oxidation of β-hydroxybutyrate to acetoacetate. The experiments do not allow any conclusions on the mechanism of the formation of citrate in the presence of oxaloacetate and acetoacetate.

A number of similar experiments were carried out (before the tracer work of Buchanan *et al.* (1945) decided the question conclusively) with the intention of throwing light on the mechanism of the increased citric acid formation in the presence of oxaloacetate and acetoacetate, especially on the conflicting views expressed by Breusch (1943*a*, *b*) and by Wieland & Rosenthal (1943) on the one hand and by ourselves (Krebs & Eggleston, 1945) on the other. If our earlier view that acetoacetate does not take a direct part in the formation of citrate were to apply also to aerobic conditions, the sum of the reaction products arising from oxaloacetate, i.e. the acids of the tricarboxylic cycle, including pyruvic acid, would be limited by the amount of carbon added in the form of oxaloacetate. Each molecule of tricarboxylic acid, α-keto- and α-hydroxy-glutarate and the bulk of the succinate molecules would require 2 molecules of oxaloacetate and each molecule of fumarate, malic and pyruvate would require 1 molecule of oxaloacetate. According to Breusch and Wieland & Rosenthal, only 1 molecule of oxaloacetate would be needed for each molecule of acid because acetoacetate would provide part of the carbon skeleton of the tricarboxylic acids, of α-keto- and α-hydroxyglutarate and of part of succinate.

Calculations based on five experiments are shown in Table 6. According to the last column more 'oxaloacetate equivalents' were recovered in some experiments than were added; this discrepancy was always greater when acetoacetate was present. The latter observation argues against our previous contention and suggests that acetoacetate contributes towards the formation of the acids of the tricarboxylic cycle. The results in Table 6 however can hardly be taken as decisive, as the effects in question are relatively small and the errors of analysis and uncertainties in the calculations may be considerable; the errors are the aggregate from six analyses and the uncertainties arise from the question how much succinate is formed by reduction and how much by oxidation from oxaloacetate. In aerobic experiments, moreover, some succinate was presumably oxidized to fumarate though this reaction was inhibited during the initial stages of incubation owing to the inhibition of succinic dehydrogenase by oxaloacetate.

This line of approach was not continued when the results of the tracer work of Buchanan *et al.* (1945)

Table 6. *Comparison of the amounts of oxaloacetate added and the amounts of substances arising from oxaloacetate*

(The quantities of metabolites arising from oxaloacetate are expressed in 'oxaloacetate equivalents'. For the purpose of calculating the equivalents, the tricarboxylic acids, α-keto- and α-hydroxy-glutaric acids are taken as requiring two oxaloacetate equivalents/molecule, fumaric, malic and pyruvic acids as requiring one equivalent. Succinic acid was taken as requiring two equivalents because in the presence of acetoacetate most of the succinate is known to be formed by oxidation from α-ketoglutarate (Krebs & Eggleston, 1945). All data refer to 48 ml. minced tissue suspension containing 1 part of tissue for 9 parts of phosphate saline.)

Tissue	Substrates added (μl.)	Aerobic or anaerobic	Period of incubation (min.)	Oxaloacetate removed (μl.)	Acetoacetate 'oxidized' (μl.)	Oxaloacetate equivalents recovered (μl.)	Balance of oxaloacetate (μl.)
Sheep kidney cortex	(*a*) Oxaloacetate (21,500)	N_2	40	20,290	—	17,530	+2,760
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,460)			19,650	2,730	19,850	− 200
Sheep heart	(*a*) Oxaloacetate (21,500)	N_2	40	21,170	—	22,540	−1,370
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,750)			19,320	2,330	23,530	−4,210
Sheep kidney cortex	(*a*) Oxaloacetate (10,750)	N_2	30	10,280	—	11,480	−1,200
	(*b*) Oxaloacetate (10,750) plus acetoacetate (22,600)			9,320	1,460	10,820	−1,500
Sheep heart	(*a*) Oxaloacetate (21,500)	O_2	40	17,450	—	15,210	+2,240
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,270)			16,370	4,632	13,700	+2,670
Sheep kidney cortex	(*a*) Oxaloacetate (10,750)	O_2	30	10,480	—	9,980	+ 500
	(*b*) Oxaloacetate (10,750) plus acetoacetate (22,600)			9,850	3,850	13,090	−3,240

became available, as this work provided direct evidence for the participation of acetoacetate in the formation of the tricarboxylic acids and α-ketoglutarate. The data reported in their paper are in accordance with this conception.

Metabolism of DL-*threo- and* DL-*erythro*-1:2-*dihydroxybutyric acids in sheep kidney cortex*

If it is agreed that acetoacetate provides part of the tricarboxylic acid and α-ketoglutarate molecule, there remains the problem to be solved of the intermediary stages of the formation of these substances from oxaloacetate and acetoacetate.

The rapid reduction of acetoacetate to β-hydroxybutyrate, even in the presence of O_2, suggests the possibility that the reduction is a stage in the final breakdown of acetoacetate. This step might then be followed by an oxidation of β-hydroxybutyrate to 1:2-dihydroxybutyric acid (see Weil-Malherbe, 1938; Haarmann & Schroeder, 1938; Hoff-Jørgensen, 1940). There are apparently conflicting statements in the literature on the metabolism of 1:2-dihydroxybutyric acids. Weil-Malherbe (1938) found that kidney does not remove the erythro form, but oxidizes the threo form to α-hydroxyacetoacetate, a reaction to which he attaches no physiological significance because α-hydroxyacetoacetate is not further metabolized. Hoff-Jørgensen reported on the other hand that the erythro form is rapidly destroyed after intravenous injection, whilst the threo form was stable under the same conditions. In view of these discrepancies the question was reexamined. The results essentially confirm Weil-Malherbe's findings (Table 7). The erythro form was not attacked by sheep kidney; the threo form caused a slight increase in the O_2 uptake and a slight decrease of the concentration equivalent to 0·8 ml. 0·01 N-iodine. This is accounted for, according to Weil-Malherbe, by oxidation to α-hydroxyacetoacetate. Thus neither the threo nor the erythro forms can be intermediates in the removal of ketone bodies by sheep kidney cortex.

Sequence of formation of α-ketoglutaric and tricarboxylic acids

From Ochoa's work (1945) it is known that the reaction *iso*citrate → α-ketoglutarate + CO_2 is reversible in some tissues. Hence the system

$$\text{citrate} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \textit{cis}\text{aconitate} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \textit{iso}\text{citrate} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \alpha\text{-ketoglutarate} + CO_2 \quad (1)$$

is completely reversible and the simultaneous occurrence of α-ketoglutarate and the tricarboxylic acids, as the result of an interaction between oxaloacetate and acetoacetate (or between other metabolites), may be due to the system (1) reacting from left to right, or in the reverse order (see also Wood, 1946). The following experiments were designed to throw light on this question. If the sequence is

$$\text{oxaloacetate} + \text{acetoacetate} \rightarrow \text{tricarboxylic acid} \rightarrow \alpha\text{-ketoglutarate} \quad (2)$$

tricarboxylic acids should yield α-ketoglutarate at least as rapidly as a mixture of oxaloacetate and acetoacetate. Experiments in Table 8 show that this is the case in sheep kidney. Various substrates and substrate combinations were added to malonate-poisoned tissue, and the sum of α-ketoglutarate and succinate was measured after aerobic incubation. The reductive formation of succinate from fumarate is small under the conditions of the experiment, and the sum of α-ketoglutarate and succinate may therefore be taken as a measure of the formation of α-ketoglutarate.

It will further be seen from Table 8 that pyruvate, acetate and acetoacetate cause some formation of α-ketoglutarate. In Exp. III the increase caused by combination of pyruvate + acetate and pyruvate + acetoacetate is somewhat larger than the sum of the individual increases, but in every case the tricarboxylic acids yield α-ketoglutarate more rapidly than other substrates. The results are thus in accord-

Table 7. *Metabolism of* DL-*threo- and* DL-*erythro*-1:2-*dihydroxybutyric acids in sheep kidney cortex*

(Minced tissue suspended in 9 parts phosphate saline. Incubation 90 min. Determination of dihydroxybutyric acid with periodate according to Weil-Malherbe (1938); the iodine equivalent of the added substrate was 16 ml. 0·01 N-iodine at the beginning; 1 ml. 0·01 N-I_2 = 112 μl. DL-dihydroxybutyrate.)

Substrate added (final conc. 0·02 M)	Q_{O_2}	Iodine equivalent (ml. 0·01 N-I_2) of 4 ml. suspension: At end of incubation	Corrected for 'blank'	Change during incubation
—	−12·4	5·9	—	—
Erythro form	−12·3	23·9	18·0	+2·0
Threo form	−13·0	21·1	15·2	−0·8

ance with the sequence (2). On the other hand, the following experiments exclude the sequence oxaloacetate + acetoacetate → α-ketoglutarate → tricarboxylic acid. Suspension of sheep kidney was anaerobically incubated with various substrate combinations in a medium containing 0·02M-$NaHCO_3$ and in equilibrium with 5% CO_2 in the gas phase. Table 9 shows that α-ketoglutarate yields only traces of citrate, less than 10% of the amount formed from a combination of oxaloacetate and acetoacetate.

measured with the help of a procedure developed in this laboratory (Krebs & Eggleston, 1944). The interpretation of these measurements is based on a characteristic property of aconitase: if citrate arises in the presence of aconitase (from a precursor other than *cis*aconitate or *iso*citrate) 10·5% will gradually be converted into a mixture of *cis*aconitate and *iso*citrate (Krebs & Eggleston, 1943; Martius & Leonhardt, 1943). If, however, *cis*aconitate arises according to scheme (3) a large proportion (up to 25%)

Table 8. *Rate of formation of α-ketoglutarate from various precursors in sheep kidney cortex*

(Minced tissue suspended in 9 parts phosphate saline pH 7·4 containing 0·005M-malonate. O_2. Final substrate concentrations in Exp. 1: 0·01M except oxaloacetate which was 0·02M; in Exps. II–IV 0·02M. The data refer to 4 ml. suspension; 'α-ketoglutarate + succinate' is the amount of succinate found in the solution after treatment with acid permanganate.)

		α-Ketoglutarate + succinate formed (μl.) in presence of											
Exp.	Period of incubation (min.)	Nil	Citrate	*Cis*-aconitate	Aceto-acetate	Citrate + aceto-acetate	Pyru-vate	Pyruvate + aceto-acetate	Oxalo-acetate	Oxalo-acetate + aceto-acetate	Oxalo-acetate + pyruvate	Acetate	Pyruvate + acetate
	30	59	463	—	87	471	91	110	342	464	356	—	—
II	40	91	492	405	—	—	183	—	—	—	—	93	235
III	40	46	398	—	88	—	109	188	—	—	—	80	182
IV	20	90	—	—	103	—	137	147	—	—	—	—	—
	40	77	—	—	109	—	200	236	—	—	—	—	—

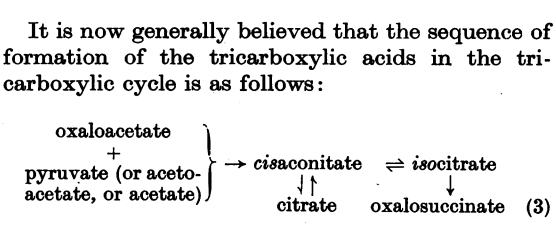
Table 9. *Rate of citrate formation in sheep kidney cortex from various precursors*

(Minced tissue suspended in 9 parts phosphate bicarbonate saline. 5% CO_2 in N_2. Substrate concentrations: 0·02M except acetoacetate which was 0·01M. Period of incubation 30 min. The data refer to 4 ml. suspension.)

Substrates added	Nil	α-Keto-glutarate	α-Keto-glutarate + aceto-acetate	Oxalo-acetate	Oxalo-acetate + aceto-acetate	Pyruvate + aceto-acetate	α-Keto-glutarate + oxalo-acetate + aceto-acetate
μl. citrate formed	Nil	23	23	119	298	23	258

Order of formation of tricarboxylic acids

It is now generally believed that the sequence of formation of the tricarboxylic acids in the tricarboxylic cycle is as follows:



This scheme rests chiefly on the work of Wood *et al.* (1941), Evans & Slotin (1941), Buchanan *et al.* (1945) and Weinhouse *et al.* (1945) which showed that citrate cannot be an intermediate on the main path leading from oxaloacetic acid to oxalosuccinic acid. Further evidence supporting this scheme is supplied by the experiments described in this section in which the formation of citrate on the one hand and of the sum of *cis*aconitate and *iso*citrate on the other was

will be converted into *iso*citrate during the early stages of the reaction. In the later stages *iso*citrate (if it is not dehydrogenated) may be reconverted into *cis*aconitate and thence into citrate until an equilibrium containing 89·5% citrate is established (Martius, 1938; Krebs & Eggleston, 1943). Therefore, (*a*) if citrate were the primary product of interaction between oxaloacetate and its partner, the sum of *cis*aconitate and *iso*citrate could never exceed 10·5% of the tricarboxylic acids present (but may be less if the reaction is interrupted before equilibrium is established, or if *iso*citrate is dehydrogenated more rapidly than it is formed); (*b*) if *cis*aconitate is produced according to scheme (3) the sum of *cis*aconitate and *iso*citrate may exceed 10·5% of the total tricarboxylic acids present provided that the reaction is interrupted before equilibrium is established (but may be less if *iso*citrate is dehydrogenated more rapidly than it is formed).

It follows that if the sum of *cis*aconitate and *iso*citrate is found to exceed the limit of 10·5% this

may be taken as conclusive evidence against the scheme (4) and as direct support of scheme (3).

$$\left.\begin{array}{c}\text{oxaloacetate}\\+\\\text{pyruvate, or}\\\text{acetoacetate or}\\\text{acetate}\end{array}\right\} \rightarrow \text{citrate} \rightleftharpoons \textit{cis}\text{aconitate}$$

$$\rightleftharpoons \textit{iso}\text{citrate} \rightarrow \text{oxalosuccinate} \quad (4)$$

Table 10 shows the results of four experiments in which the tricarboxylic acids were estimated under varying conditions. The incubation periods were relatively short because it was intended to interrupt the reaction before an equilibrium between the three tricarboxylic acids could be established. In several experiments the amounts of *cis*aconitate + *iso*citrate far exceeded the critical figure of 10·5 %, the highest value being 49 %. The fact that the ratio

$$\frac{\textit{cis}\text{aconitate} + \textit{iso}\text{citrate}}{\text{total tricarboxylic acids}}$$

shows great variations with the experimental conditions is not surprising, because this ratio is the resultant of the rates of at least six reactions (indicated by the arrows in scheme 3) each of which varies with the conditions. Variability of the ratio does not invalidate the conclusion that the results of the experiments in Table 10 support scheme 3.

Note on non-enzymic condensation reactions of oxaloacetate. Breusch (1941) noted that neutral solutions of oxaloacetate may contain substances which simulate citric acid in analytical methods based on the formation of pentabromoacetone, and that the quantities of interfering substances increased on addition of calcium salts. Our own experiments confirmed this observation, and furthermore showed that substances arise by non-enzymic reactions of oxaloacetate, which behave like *iso*citrate and *cis*aconitate, forming bromo compounds after incubation with aconitase resembling pentabromoacetone. All substances interfering with the determination of citrate and of *cis*aconitate plus *iso*citrate are however quantitatively removed by boiling the acidified solution before treatment with KBr and $KMnO_4$, an obligatory step in the citric acid determination.

Effect of $BaCl_2$ *on the yields of citrate*

Wieland & Rosenthal (1943) and Weinhouse *et al.* (1946) have found that the yields of citrate from a mixture of oxaloacetate and acetoacetate increase if $BaCl_2$ (about 0·01 M) is added to the medium. Data given in Table 11 confirm this finding. Wieland & Rosenthal offer no explanation for the cause of this effect. On the basis of the following scheme one would expect the yield of citrate to rise if a specific inhibitor reduced the rates of (*b*) or (*d*) without major effects on the rates of (*c*). In order to test whether barium ions inhibit specifically (*b*), aconitase (minced sheep heart muscle) was incubated with *cis*aconitate in the presence and absence of

Table 10. *Formation of citrate,* cis*aconitate and* iso*citrate in minced sheep kidney cortex*

(Tissue suspended in 9 parts medium. The data refer to 24 ml. suspension. The media did not contain Ca ions; pH in all cases 7·4.)

Exp. no.	Medium	Gas	Substrate added (final concentrations)	Period of incubation (min.)	Citrate formed (μl.)	*cis*Aconitate + *iso*citrate formed (μl.)	*cis*Aconitate + *iso*citrate as % of total tricarboxylic acids
I	Phosphate saline	O_2	0·01 M-Oxaloacetate	5	128	47	26·8
			0·01 M-Oxaloacetate	10	227	90	28·4
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	5	229	57	19·9
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	10	350	268	43·5
II	Phosphate-bicarbonate saline	5% CO_2 in O_2	0·02 M-Oxaloacetate, 0·02 M-pyruvate	30	1600	1560	49·4
			0·02 M-Oxaloacetate, 0·02 M-pyruvate, 0·01 M-acetoacetate	30	3200	~Nil	—
III	Phosphate saline	O_2	0·01 M-Oxaloacetate	10	190	22	10·4
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	10	415	47	10·2
IV	Phosphate-bicarbonate saline	5% CO_2 in O_2	0·02 M-Oxaloacetate	20	316	~Nil	—
			0·02 M-Oxaloacetate, 0·02 M-pyruvate	20	580	~Nil	—
			0·02 M-Oxaloacetate, 0·01 M-acetoacetate	20	1000	430	30·1
			0·02 M-Oxaloacetate, 0·01 M-acetoacetate, 0·02 M-pyruvate	20	1240	124	9·1

$BaCl_2$ and the rates of formation of *iso*citrate and citrate were measured.

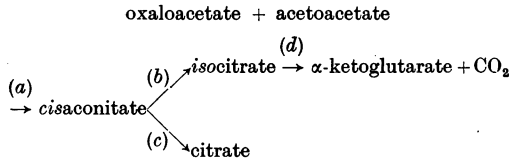


Table 11. *Effects of* $BaCl_2$ *on yields of citrate from oxaloacetate and acetoacetate*

(Minced sheep kidney cortex suspended in 9 parts phosphate-bicarbonate saline. Period of incubation 30 min.; 5% CO_2 in O_2; 0·02M-oxaloacetate; 0·01M-acetoacetate. The data refer to 4 ml.)

	Without $BaCl_2$	With 0·02M-$BaCl_2$
Oxaloacetate removed (μl.)	1310	1200
Acetoacetate reduced (μl.)	72	53
Acetoacetate 'oxidized' (μl.)	258	329
Citrate formed (μl.)	162	344
L-Malate formed (μl.)	189	169

Table 12 demonstrates the phenomenon discovered by Martius (1938) that during the early stages of incubation *iso*citrate is formed more rapidly than citrate, and that *iso*citrate is later reconverted to citrate. $BaCl_2$ (0·02M) slows down both reactions (*b*) and (*c*) a little. At 10 min. the inhibition of (*b*) is 17%, of (*c*) 28%. Thus the increased yield of citrate from oxaloacetate and acetoacetate cannot be explained by a specific inhibition of reaction (*b*).

With and without barium chloride the maximum yields of *iso*citrate are 23% of the added *cis*aconitate, whilst the final yield is only 5%. Probably owing to the conversion of some *cis*aconitate into the *trans* form during the prolonged incubation the total yield of *cis*aconitate and *iso*citrate was less than that obtained with more powerful aconitase preparations in short-term experiments (Krebs & Eggleston, 1943).

As for effects of barium on reaction (*d*) (the oxidative removal of *iso*citrate), Table 13 indicates that barium ions inhibit the O_2 uptake of muscle without citrate (see Thunberg, 1937), but citrate increases the oxygen uptake also in the presence of Ba and the rate of removal of citrate is only slightly reduced by Ba. This effect is too small to account for the increased yields of citrate and by the method of elimination one is therefore led to the assumption that the rate of *cis*aconitate formation (reaction *a*) is accelerated in the presence of barium ions. Such an

Table 12. *Effect of* $BaCl_2$ *on aconitase*

(4·2 g. frozen minced sheep heart suspended in a total volume of 100 ml.; 0·025M-phosphate buffer, pH 7·4; 0·1M-*cis*aconitate = 10 mmol. 40°. At intervals, 10 ml. solutions were removed for analysis and the reaction was stopped by addition of 1·25 ml. glacial acetic acid. *iso*Citrate was determined polarimetrically in 9 ml., citrate in 0·5 ml. The quantities of *iso*citrate and citrate are expressed in mmol.)

	No $BaCl_2$ added		0·02M-$BaCl_2$	
Time (min.)	*iso*Citrate (mmol.)	Citrate (mmol.)	*iso*Citrate (mmol.)	Citrate (mmol.)
0	0	0	0	0
5	1·17	—	1·03	—
10	1·82	1·70	1·52	1·23
20	2·26	—	1·93	—
30	2·21	3·39	2·17	2·02
40	1·93	—	2·28	—
60	1·42	4·65	2·23	3·12
90	0·91	—	1·94	—
120	0·67	6·61	1·72	4·58
180	0·53	6·58	1·41	5·24
24 hr.	0·56	6·79	0·51	6·80

Table 13. *Effect of* $BaCl_2$ *and the composition of saline medium on* O_2 *uptake and citrate removal in sheep kidney cortex*

(Minced sheep kidney suspended in 9 parts medium. 40°. 60 min. Medium I = phosphate saline, pH 7·4; medium II = 45 ml. 0·9% NaCl + 4·8 ml. M-$NaHCO_3$. (Breusch's (1943) medium). O_2.)

	Medium I				Medium II			
Substances added to medium	Nil	0·005M-Citrate	0·01M-$BaCl_2$	0·005M-Citrate, 0·01M-$BaCl_2$	Nil	0·005M-Citrate	0·01M-$BaCl_2$	0·005M-Citrate, 0·01M-$BaCl_2$
O_2 uptake (μl.)	1290	1790	830	1150	193	310	351	339
Citrate removed (μl.)	—	432	—	355	—	106	—	96

acceleration is feasible bearing in mind that a number of oxidative reactions in respiring cells compete with, and thus inhibit, each other; lactate, e.g., inhibits the oxidation of L-amino acids (see Krebs, 1935). It is feasible that barium ions inhibit a reaction which normally compete with, and thus slows down, the formation of citrate. This would result in an increased rate of citrate formation.

According to Breusch (1943*a*, *b*) the presence of bicarbonate in the medium, without CO_2 in the gas phase, also increases the yields of citrate from oxaloacetate. Table 13 shows that the oxygen uptake and citrate removal are greatly reduced in Breusch's medium. The increased yields of citrate in this case might thus be explained by the assumption that in this medium, which is unphysiologically alkaline, the formation of citrate is less inhibited than its removal.

DISCUSSION

Acetoacetate and the tricarboxylic acid cycle. The facts that the C_4-dicarboxylic acids promote the aerobic removal of acetoacetate and that acetoacetate increases the total yield of the acids of that cycle in the presence of oxaloacetate are in accordance with the conception, already safely established by tracer work, that acetoacetate is metabolized through the tricarboxylic cycle. So far there is no conclusive evidence suggesting alternative pathways of acetoacetate breakdown.

The main gap in the present knowledge of acetoacetate metabolism concerns the reactions leading from acetoacetate and oxaloacetate to *cis*aconitate. It is noteworthy that these reactions appear to require the presence of O_2, although the balance sheet of the reaction

acetoacetate + oxaloacetate → *cis*aconitate

does not represent an oxidation.

Our previous arguments (Krebs & Eggleston, 1945) against the hypothesis of Breusch (1943*a*, *b*) and of Wieland & Rosenthal (1943) were based on anaerobic experiments, where practically all the acetoacetate removed in the presence of oxaloacetate was accounted for as β-hydroxybutyrate. We pointed out that Breusch and Wieland & Rosenthal had supplied no evidence that under their experimental conditions acetoacetate was removed by a reaction other than reduction and that an alternative explanation was available for the increased yields of citrate. These arguments are now superseded by the tracer work of Buchanan *et al.* (1945) and Weinhouse *et al.* (1945, 1946).

Mechanism of condensation. The following considerations are offered as a contribution to the unsolved problem of the mechanism whereby oxaloacetate condenses with a second molecule to form the 6-carbon skeleton of the tricarboxylic acids. The tracer experiments have made it clear that citrate cannot be an intermediate because the isotopic carbon introduced as $CH_3.{}^{13}CO.CH_2.{}^{13}COOH$ was unsymmetrically distributed over the two carboxyl groups of α-ketoglutarate, the major part being in the γ-carboxyl. These facts are in accordance with the view that *cis*aconitate is the primary product of condensation provided that the reaction *cis*aconitate → *iso*citrate → α-ketoglutarate is very rapid in comparison with the reaction *cis*aconitate → citrate. Experimental evidence supporting this assumption has been presented previously (Krebs, 1943).

By analogy with well known organic chemical reactions a possible mechanism of formation of *cis*aconitate would be an aldol condensation of the type of the Perkin reaction or Knoevenagel reaction:

$$R.CHO + CH_3COONa \rightarrow R.CH{:}CH.COONa.$$

Applied to oxaloacetate and an acetate residue this type of condensation would yield a *cis*aconitic acid with the double bond in the 2:3 position to the carboxyl of the acetate residue. This would form an α-ketoglutarate whose carboxyl adjacent to the α-ketonic group is derived from the carboxyl of the acetate residue. The tracer experiments show that this is not the case. No double bond is formed as a result of the enzymic condensation. The double bond in *cis*aconitic acid, as already assumed by Wood *et al.* (1941), must be derived from that of the *enol*oxaloacetate (hydroxymaleic acid).

SUMMARY

1. The aerobic removal of acetoacetate by minced sheep heart and kidney cortex was investigated and a number of metabolites formed in the presence of acetoacetate were determined.

2. Manometric methods were developed for the determination of pyruvate in the presence of oxaloacetate and of oxaloacetate in the presence of pyruvate plus acetoacetate. In elaborating these methods it was noted that oxaloacetate gives variable yields of CO_2 with yeast carboxylase and that mixtures of acetoacetate and α-ketonic acids (pyruvate, α-ketoglutarate) behave towards Al ions like dicarboxylic β-ketonic acids, which suggests that these mixtures contain β-ketonic acids formed by aldol condensation.

3. In heart and kidney cortex some of the added acetoacetate was rapidly reduced to β-hydroxybutyrate even under aerobic conditions, whilst another fraction underwent oxidation. The rate of oxidation was increased by oxaloacetate and by fumarate. Data on the rates of reduction and oxidation of acetoacetate under varying conditions are given.

4. Nine metabolites (oxaloacetate, acetoacetate, pyruvate, β-hydroxybutyrate, succinate, fumarate,

L-malate, citrate, α-ketoglutarate) found in solutions as a result of the interaction between acetoacetate and oxaloacetate were determined in aerobic and anaerobic experiments. The most notable effect of oxygen on the yields of various substances was a higher yield of citrate. Calculations based on the yields suggest that acetoacetate contributes towards the formation of the acids of the tricarboxylic cycle, a conclusion now firmly established by the tracer work of Buchanan *et al.* and of Weinhouse *et al.*

5. Erythro-1:2-dihydroxybutyric acid was not metabolized by sheep kidney cortex whilst the threo form was slowly oxidized. In accordance with Weil-Malherbe's findings, the slowness of the oxidation excludes this substance as an intermediate in the removal of ketone bodies.

6. Measurements of the rates of α-ketoglutarate formation from various substrates and substrate combinations showed that the tricarboxylic acids yield α-ketoglutarate more rapidly than any other substrate. This supports the assumption that α-ketoglutarate arises via the tricarboxylic acids when formed in the presence of oxaloacetate and acetoacetate (or pyruvate). The rate of citrate formation via carboxylation of α-ketoglutarate (Ochoa, 1945) was relatively slow in sheep kidney cortex.

7. The sum of *cis*aconitate plus *iso*citrate was found to exceed 10·5 % of the total tricarboxylic acids (maximum 49 %) under suitable conditions. It is pointed out that this finding excludes the sequence (4) (p. 302) and supports the sequence (3) (p. 301), confirming conclusions earlier drawn from tracer work.

8. It was confirmed that barium (Wieland & Rosenthal, 1943) and bicarbonate (Breusch, 1943*a*, *b*) ions increase the yield of citrate from oxaloacetate and acetoacetate. Possible causes of this effect were investigated.

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The Isolation of Hydroxylysine Picrate from a Gelatin Hydrolysate

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Schryver, Buston & Mukherjee (1925) claimed the isolation, from isinglass hydrolysate, of an 'oxylysine' to which they attributed the formula $C_6H_{14}O_3N_2$; but they were unable, on repeated attempts, to prepare a crystalline picrate. Van Slyke & Hiller (1921) reported the presence of hitherto unknown material in the basic amino-acid fraction of hydrolyzed gelatin; this base was isolated by Van Slyke, Hiller, Dillon & MacFadyen (1938) who ascribed to it the formula of a hydroxylysine. After removal of arginine and histidine, these workers freed the 'lysine fraction' from monoamino-acids, and then removed the greater portion of the lysine by the addition of sufficient picric acid to combine with three quarters of the amino nitrogen present. A picrate of composition $C_6H_{14}O_3N_2 . C_6H_3O_7N_3$, which melted at 225° when heated slowly, was eventually isolated from the mother liquor. Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940) and Van Slyke, Hiller & MacFadyen (1941)