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EXPLANATION OF PLATES 1-4

PLATE 1

Fig. 2. Untreated cell walls of *Chlorella*. Microfibrils can be seen and the undersurface of the wall is visible at the breakage cleft.

Fig. 4. Cell walls treated with 3% NaOH for 14 hr. at room temperature. The cell wall has completely disintegrated, giving bundles of microfibrils with very little matrix material visible.

Fig. 5. Cell walls treated with ethanolamine for 4 hr. at 25°, showing microfibrils.

Fig. 7. Cell walls treated with 0.5% NaOH for 25 min. at room temperature and then snail digestive enzyme at 25° for 14 hr. No microfibrils are visible and although the wall is very thin it still retains the cell shape.

PLATE 2

Fig. 3. Cell walls treated with NaOH at room temperature: (a) 0.5% NaOH for 5 min.; (b) 0.5% NaOH for 25 min.; (c) 0.5% NaOH for 25 min.; (d) 0.5% NaOH for 30 min.; (e) 3% NaOH for 30 min. Microfibrils lying in two

principal directions at right angles to one another can be clearly seen. The granular material of the matrix is visible. In (e) the wall has lost its form and is disintegrating into microfibrils.

PLATE 3

Fig. 6. Cell walls treated with snail digestive enzyme for 14 hr. at 25°, showing rough granular surface and absence of microfibrils.

Fig. 9. Section of whole cell. The appearance of layers in the cell wall is visible. Compare with Plate 4, Fig. 8.

Fig. 11. Section of cell walls treated similarly to those shown in Plate 2, Fig. 3c. The walls have swollen and the microfibrils are visible as a diffuse material throughout the thickness of the sections.

PLATE 4

Fig. 8. Sections of untreated cell walls showing ribbon-like appearance and characteristic curling at the breakage cleft. Compare with Plate 1, Fig. 2 and Plate 3, Fig. 9.

Fig. 10. Section of cell walls treated similarly to those shown in Plate 3, Fig. 6. The appearance of two layers in the wall is very apparent.

Phosphorolysis of Citrulline by Mammalian Liver: the Effect of a Bacterial Activator

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In a previous paper (Krebs, Eggleston & Knivett, 1955) it was shown that washed suspensions of *Escherichia coli* N.C.I.B. 8571 accelerate the phosphorolysis of citrulline by mammalian liver extracts. The bacterial cells contained ornithine decarboxylase, and most of the acceleration could be ascribed

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to the removal of ornithine, which acted as an inhibitor of the phosphorolysis. However, an accelerating effect remained even when the activity of ornithine decarboxylase was abolished by hydroxylamine. From this it was concluded that this strain of *E. coli* contained an additional substance (or 'factor') which promotes the phosphorolysis of citrulline.

According to Jones, Spector & Lipmann (1955*a*) carbamyl phosphate is an intermediate in the synthesis of citrulline, and it may therefore be expected that the phosphorolysis of citrulline primarily leads to a formation of ornithine and carbamyl phosphate, and further that carbamyl phosphate, like ornithine, inhibits phosphorolysis. If this were the case, phosphorolysis would be accelerated by agents which promote the hydrolysis of carbamyl phosphate. This paper is concerned with the examination of the question whether the bacterial 'factor' is a carbamyl phosphatase.

EXPERIMENTAL

Carbamyl phosphate. This was prepared as the dilithium salt according to Jones, Spector & Lipmann (1955*b*). The salt was analysed for phosphate and NH_3 before and after complete hydrolysis in 0.01 N-HCl at 100° for 1 min. After correction for free phosphate (5%) and free NH_3 (1%) the purity of the salt was calculated to be of the order of 97–98%. Ammonia was determined by direct nesslerization. When the salt was added to H_2SO_4 solution (2%, v/v), the yield of CO_2 , measured manometrically, was only 92% of the expected value. The reason for this low yield is obscure.

Buffers. $\beta\beta$ -Dimethylglutarate buffers were made according to Stafford, Watson & Rand (1955), 'tris maleate' buffers according to Gomori (1955), sodium phosphate buffers from mixtures of Na_2HPO_4 and NaH_2PO_4 , citrate buffers from sodium citrate and NaOH. A glass electrode and a Pye pH meter were used to measure the pH of these buffers after dilution to the concentrations used in the experiments.

Liver preparations. Rat or guinea-pig liver was broken up in a Waring Blendor with 4 parts of water; this suspension was dialysed against ice-water for 24 hr. and stored at -15° . A purified preparation of the system responsible for the phosphorolysis of citrulline was made from an acetone-dried powder of guinea-pig liver, which was extracted with 25 vol. of water and subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation according to Krebs *et al.* (1955). The precipitate formed after 60% saturation was dissolved in water amounting to one-fifth of the volume of the supernatant and dialysed against ice-water for 24 hr.

A precipitate formed during the dialysis was removed. The supernatant contained the enzyme system. It was stored at -15° ; on thawing a precipitate appeared which was evenly suspended before pipetting the material.

Other animal tissues. One part of freshly obtained tissue, chilled in iced saline, was disintegrated in a stainless-steel Potter-Elvehjem homogenizer with 10 parts of ice-water.

Escherichia coli. The strains used and their growth have been described by Eggleston (1957).

Estimation of 'factor' activity. The 'factor' activity is defined as the increase in CO_2 evolution from citrulline in the presence of liver enzyme and phosphate, when both *E. coli* preparations and hydroxylamine are added. The CO_2 evolution was measured manometrically in a Warburg apparatus. The main compartment of the flasks usually contained 0.5 ml. of liver enzyme, 0.25–1.0 ml. of other enzyme preparations (*E. coli* or animal tissues), 0.5 ml. of M-sodium phosphate buffer, pH 6.8, and 0.25 ml. of 0.1 M-hydroxylamine-HCl. The side arm contained 0.5 ml. of 0.5 M-DL-citrulline and the gas space N_2 . The total volume of fluid was 2.5–3.0 ml. The citrulline was added after thermal equilibration for 15 min. at 40°. In some experiments 0.5 ml. of 10% (v/v) H_2SO_4 was added from a second side arm at the end of the incubation period in order to release CO_2 bound by the buffer. Corrections were made for the small CO_2 evolution which occurred in controls containing *E. coli* and liver or *E. coli* and citrulline. Hydroxylamine was present in these controls.

Estimation of 'carbamylphosphatase' activity. The rate of decomposition of carbamyl phosphate was measured either by manometric determination of the rate of CO_2 evolution or by colorimetric phosphate determination. In the manometric test the side arm contained 0.2–0.4 ml. of 0.05 M-carbamyl phosphate, freshly dissolved, and the main compartment buffer and the enzyme preparation. The temperature was 25°. When the enzyme activity was measured by inorganic phosphate determination, the incubation was carried out in centrifuge tubes. The total volume was 1 ml. and the final concentrations of substrate and buffer the same as in the Warburg flasks. The reaction was stopped by the addition of 1.5 ml. of 5% (w/v) ice-cold trichloroacetic acid and the protein precipitate was immediately spun down at 0° for 3 min. A 1 ml. sample of the supernatant was removed for phosphate determination by the method of Berenblum & Chain (1938) or by the method of Lowry & Lopez (1946) as modified by Peel, Fox & Elsdon (1955).

Table 1. Relative 'factor' activities in fractions of *Escherichia coli* N.C.I.B. 8571

Enzymic assay was carried out as described in the Experimental section. $Q_{\text{CO}_2} = \mu\text{l. of CO}_2$ evolved in 60 min./mg. dry wt.

No.	Fraction	Q_{CO_2}
	Cell suspension	4.6
	Cell-free extract	4.0
1	Ppt. from MnCl_2 addition, dialysed	
	{ insoluble fraction	0.6
	{ soluble fraction	8.8
2	Ppt. from 40% saturation of supernatant with $(\text{NH}_4)_2\text{SO}_4$, dialysed	
	{ insoluble fraction	1.2
	{ soluble fraction	0
3	Ppt. from 60% saturation of supernatant with $(\text{NH}_4)_2\text{SO}_4$, dialysed	
	{ insoluble fraction	4.7
	{ soluble fraction	6.1
4	Ppt. from 80% saturation of supernatant with $(\text{NH}_4)_2\text{SO}_4$, dialysed	
	{ insoluble fraction	6.8
	{ soluble fraction	60.0
5	Ppt. from final acidification with trichloroacetic acid to pH 2	5.7

Fractionation of 'factor' activity in Escherichia coli N.C.I.B. 8571. Thick, cream-like suspensions of *E. coli* cells from 5 l. of growth medium were crushed in the Hughes press (Hughes, 1951) at -15 to -25° and extracted with 0.15M-NaCl at 2° for 24 hr. The volume of the NaCl solution was 50 times that of the dry weight of the crushed cells. Uncrushed cells, cell debris and undissolved material were centrifuged off at $24\,000\text{ g}$ for 30 min. To the supernatant was added one-twentieth of a volume of M-MnCl_2 and the precipitate, consisting mainly of nucleoproteins, was removed by centrifuging. The supernatant was then subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation, starting with 40% saturation. The precipitates formed at 40, 60 and 80%, and after final acidification with trichloroacetic acid to pH 2, were suspended or dissolved in one-fifth of the volume of the corresponding supernatant and dialysed against ice-water for 48 hr. The fractions were then centrifuged and the soluble and insoluble sub-fractions tested for 'factor' activity. The results are given in Table 1. The most active fraction, that formed after 80% $(\text{NH}_4)_2\text{SO}_4$ saturation, was completely soluble in water, but during dialysis a precipitate was formed. This contained only a minor fraction of the activity. Although all stages of the fractionation were carried out below 5° , there was a loss of 60–85% of the original total activity. The most active extracts were stored at -15° for further use.

RESULTS

Properties of the 'factor' system. The 'factor' activity increased with the citrulline concentration over the range tested (20–100 mM), and was approximately proportional to the amount of *E. coli* preparation up to about 10 mg. dry wt. of cells/flask. With this quantity of bacteria and 0.5 ml. of the purified liver enzyme, 80 $\mu\text{l.}$ of CO_2 was formed in 60 min. at a DL-citrulline concentration of 0.1 M. The rate of the reaction was initially constant at high concentrations of DL-citrulline (0.1 M) but later gradually fell, probably because of the accumulation of ornithine. The reaction continued for several hours, an indication of the relative stability of the enzyme systems.

The pH optimum for 'factor' activity was found to be near pH 6.2 in phosphate buffer (Table 2).

Table 2. *Effect of pH on 'factor' activity*

Cups contained 0.5 ml. of liver enzyme, 0.75 ml. of the soluble pool of 'fraction 4' (Table 1) (2 mg. of protein); 0.5 ml. of M-phosphate buffer; 0.25 ml. of 0.1 M-hydroxylamine; 0.5 ml. of 0.5 M-DL-citrulline; 0.5 ml. of 10% (v/v) H_2SO_4 (second side arm). Total vol. 3.0 ml.; temp. 40° . Gas phase N_2 .

pH	CO_2 formed in 120 min. ($\mu\text{l.}$)		
	Free	Bound	Total
5.9	160	0	160
6.2	175	3	178
6.5	131	8	139
6.8	123	16	139
7.1	85	20	105
7.4	35	12	47

The optimum for the liver enzyme causing arsenolytic or phosphorolytic cleavage of citrulline is about pH 6.7 (Krebs *et al.* 1955).

Carbamyl phosphate decomposition. There was a rapid decomposition of carbamyl phosphate when incubated with purified 'factor' preparations, the rate of liberation of phosphate being equal to that of CO_2 evolution. At 40° and below pH 6.0 there was also a rapid non-enzymic decomposition of carbamyl phosphate amounting during the first hour to 20–40% of the substance present. At higher pH values and 25° the non-enzymic decomposition was only a few per cent./hr. Variations of pH from about 4.5 to 7.0 did not affect the rates of enzymic hydrolysis; at pH 4.0, 8.2 and 9.2 the rates were 73, 73 and 43% respectively of the maximum. The final amounts of CO_2 evolved on enzymic hydrolysis were identical with that released by strong acid at 40° , namely, 92% of the expected value.

According to Jones *et al.* (1955*a*) the decomposition of carbamyl phosphate proceeds in two stages, with the intermediate formation of carbamic acid:

In order to ascertain which of the two steps is catalysed by *E. coli*, carbamyl phosphate was hydrolysed to carbamate and phosphate before incubation by placing 0.25 ml. of 0.1 M-carbamyl phosphate and 0.5 ml. of 0.1 N-KOH in the side arm of a Warburg flask. The phosphate was liberated quantitatively during thermal equilibration for 10 min. at 40° ; the carbamate formed was almost completely stable under these conditions. The excess of KOH was neutralized on mixing by 0.25 ml. of 0.1 N-HCl added to the buffer in the main compartment. The decomposition of the carbamate at pH 6.0 was found to be very rapid and was not affected by the addition of purified 'factor' preparations. Thus the enzymic action is on stage (1).

Activities of 'factor' and 'carbamylphosphatase' in other strains of Escherichia coli and in rat tissues. Table 3 compares the 'carbamylphosphatase' activity of various *E. coli* preparations and of rat tissues, with their 'factor' activity, calculated per mg. dry wt. 'Carbamylphosphatase' activity was found in all these materials, but 'factor' activity was absent from strains T and 4, and only slight in strains 86, F and 8114; strain 8571 had the highest 'factor' activity. In the rat tissues tested no 'factor' activity could be detected. There is thus no parallelism between the two activities. This result dismisses the assumption that the 'factor' is a 'carbamylphosphatase'.

Effects of various substances on 'factor' and 'carbamylphosphatase' activities. The phosphorolysis of citrulline in the presence of liver and *E. coli* is accelerated by increasing concentrations of phosphate (see Krebs *et al.* 1955, Table 10). Since phosphate stimulates the bacterial ornithine decarb-

oxylase (Eggleson, 1957), the phosphate effect could be attributed to its effect on the decarboxylase, but experiments in which sufficient hydroxylamine was added to inhibit ornithine decarboxylase almost completely, showed that phosphate also stimulates 'factor' activity. Thus increasing the

Table 3. 'Factor' and 'carbamylphosphatase' activities in different strains of *Escherichia coli* and in rat tissues

'Factor' activity was measured in phosphate buffer, pH 6.8, at 40°, 'carbamylphosphatase' activity in 0.05 M-dimethylglutarate buffer, pH 5.25, at 25° with 5 mM-carbamyl phosphate as substrate and a final volume of 4.0 ml. Q_{CO_2} = μ l. of CO_2 liberated/mg. dry wt. of enzyme/hr., for the period 0–30 min.

Material tested	'Factor' activity (Q_{CO_2})	'Carbamyl-phosphatase' activity (Q_{CO_2})
<i>E. coli</i> F, washed cells	2.1	20.1
<i>E. coli</i> 4, washed cells	0	12.8
<i>E. coli</i> T, washed cells	0	10.6
<i>E. coli</i> N.C.T.C. 86, washed cells	1.0	31.8
<i>E. coli</i> N.C.I.B. 8114, washed cells	1.1	12.2
<i>E. coli</i> N.C.I.B. 8571, washed cells	5.2	29.5
<i>E. coli</i> N.C.I.B. 8571, purified extract from 80% $(NH_4)_2SO_4$ fractionation	60	270
Rat-lung homogenate	—	1.6
Rat-liver homogenate	0	5.7
Rat-kidney homogenate	0	5.8
Rat-spleen homogenate	0	13.3
Rat-brain homogenate	—	2.5
Rat-heart homogenate	—	1.9

Table 4. Effect of L-asparagine, L-aspartate and fumarate on 'factor' activity

Each cup contained 0.5 ml. of dialysed guinea-pig liver suspension; 0.5 ml. of *Escherichia coli* 8571 suspension (30 mg. dry wt.); 0.5 ml. of M-sodium phosphate buffer, pH 6.8; 0.3 ml. of 0.1 M- $NH_4OH \cdot HCl$; 0.5 ml. of 0.5 M-DL-citrulline. Final concentrations of L-asparagine, L-aspartate and fumarate were 33 mM. Total volume 3.0 ml. Gas phase N_2 . Temp. 40°. Results represent μ l. of CO_2 liberated.

Additions	Period 1 (0–60 min.)	Factor activity	Period 2 (60–120 min.)	Factor activity	Period 3 (120–240 min.)	Factor activity
None	34	—	22	—	44	—
Citrulline	88	54	54	32	85	41
Asparagine	36	—	40	—	173	—
Asparagine + citrulline	86	50	60	20	269	96
Aspartate	37	—	55	—	171	—
Aspartate + citrulline	100	63	101	46	375	204
Fumarate	68	—	110	—	197	—
Fumarate + citrulline	122	54	211	101	359	162

Table 5. Effect of pre-incubation with aspartate or fumarate on the production of an increased 'factor' activity

Conditions were as described in Table 4, except that final concentrations of L-aspartate and fumarate were 28 mM. Total vol. 3.6 ml. in N_2 at 40°. Shaken for 2 hr. before addition of citrulline from the side arm. Results represent μ l. of CO_2 liberated after the addition of citrulline.

Cup no.	...	1	2	3	4	5	6			
Additions	...	None	Citrulline	Factor activity (2 – 1)	Aspartate	Aspartate + citrulline	Factor activity (4 – 3)	Fumarate	Fumarate + citrulline	Factor activity (6 – 5)
First 30 min.		19	61	42	142	258	116	181	293	112
Second 30 min.		22	59	37	105	263	158	191	299	108
Third 30 min.		28	49	21	74	212	138	96	219	123
Fourth 30 min.		32	63	31	51	165	114	61	170	109
Fifth 30 min.		32	71	39	50	161	111	54	158	104
Sixth 30 min.		33	73	40	46	128	82	44	111	67

Table 6. *Effect of adenosine 5'-phosphate (AMP) on 'factor' activity*

Conditions were as described in Table 4 except that dialysed rat liver was used and AMP was 10 mM final concentration where added. Controls (not shown) with AMP added, but omitting either liver or *E. coli*, gave no extra CO₂ in the presence of citrulline. Results represent μ l. of CO₂ liberated.

Cup no.	...	1	2		3	4		
Additions	...	None	Citrulline	Factor activity (2-1)	AMP	AMP + citrulline	Factor activity (4-3)	AMP effect
First 30 min.		23	64	+41	103	152	+ 49	+ 8
Second 30 min.		34	67	+33	134	315	+181	+148
Third 30 min.		34	57	+23	269	464	+195	+172
Fourth 30 min.		39	63	+24	317	525	+208	+184

phosphate concentration from 16 mM to 0.2 M caused a threefold increase in the amounts of CO₂ liberated.

Adenosine 5'-phosphate (AMP), L-aspartate, L-asparagine, fumarate and glycerol were also found to increase the rate of 'factor' activity. The effects are not immediate but develop with time (Tables 4-6); the highest increases were obtained with AMP, where it was +20% after 30 min. and +420% after 2 hr. These substances had no effect on the hydrolysis of carbamyl phosphate. They all also increased the rate of CO₂ evolution in the absence of citrulline, because of the fermentation of the added substrate by *E. coli*. Citrulline has no effect on the rate of these fermentations, as measured by CO₂ production. The four named substances do not form CO₂ in the presence of the liver preparation without *E. coli*. It should be mentioned that the effects of aspartate, asparagine and fumarate were observed with sheep and guinea-pig liver, but not with rat liver; on the other hand, rat liver gave effects with glycerol and AMP.

Succinate (20 mM) and L-malate (17 mM) caused only small increases in 'factor' activity (15-30% in 2 hr.). Adenosine triphosphate (10 mM), yeast adenylic acid (10 mM), pyridoxal phosphate (10 μ g./ml.), tetraethyl pyrophosphate (10 mM) and methanol (33 mM) were without effect.

Citrate (17 mM), pyruvate (17 mM), L-glutamate (17 mM), NH₄Cl (20 mM) and carbamyl phosphate (10 mM) inhibited 'factor' activity by 100, 66, 100, 84 and 52% respectively (incubation time 120-180 min.). The inhibition by carbamyl phosphate (which underwent rapid decomposition) may have been due to the NH₃ formed. The enzymic breakdown of carbamyl phosphate by *E. coli* was not inhibited by citrulline (33 mM).

DISCUSSION

The experiments reported in this paper show that *Escherichia coli* preparations which promote the phosphorolysis of citrulline by mammalian liver extracts in the presence of hydroxylamine contain

a carbamylphosphatase, but there was no parallelism between the promotion of citrulline phosphorolysis and carbamylphosphatase activity. The factor in *E. coli* which, in addition to ornithine decarboxylase, promotes citrulline phosphorolysis, is thus unlikely to be a carbamylphosphatase. This conclusion is borne out by the fact that carbamylphosphatase preparations from animal tissues cannot replace the *E. coli* 'factor'. The nature of this 'factor' remains obscure.

The activity of the 'factor' is increased by aspartate, fumarate, adenosine 5'-phosphate and other substances, whereas the hydrolysis of carbamyl phosphate is not affected. This confirms the above conclusion.

The experiments also show that carbamyl phosphate is readily hydrolysed by enzymes present in animal tissues and in various strains of *E. coli*. Whether this hydrolysis is due to a specific enzyme or to one of the unspecific phosphatases has not been examined.

The possibility that carbamyl phosphate is an intermediate in the phosphorolysis of citrulline is not excluded by the present experiments.

SUMMARY

1. A heat-labile bacterial 'factor' (other than ornithine decarboxylase) capable of stimulating the phosphorolysis of citrulline by mammalian liver has been purified 15-fold by ammonium sulphate fractionation. The purified preparation and washed suspensions of several strains of *Escherichia coli* were tested for 'factor' activity and carbamyl phosphatase activity, because acceleration of the hydrolysis of carbamyl phosphate might account for the 'factor' activity.

2. Carbamylphosphatase activity was found in all 'factor' preparations, but there was no parallelism with 'factor' activity. The latter was absent from rat spleen, kidney and liver, although these tissues rapidly hydrolysed carbamyl phosphate, and *E. coli* N.C.I.B. 8571 was the only one of six strains which had appreciable 'factor' activity.

3. Adenosine 5'-phosphate, L-aspartate, L-asparagine, fumarate and glycerol each stimulated 'factor' activity but had no effect on the enzymic hydrolysis of carbamyl phosphate. This stimulation generally increased with time.

4. Some characteristics of the bacterial hydrolysis of carbamyl phosphate are described.

5. The results indicate that the bacterial 'factor' is not a carbamylphosphatase.

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The Formation of Acetoacetate in Homogenates of the Mammary Gland

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Apart from the liver, which is generally considered the principal site of ketogenesis (see Weinhouse, 1952; Campbell & Best, 1956), the rumen is also recognized as a source of the ketone bodies circulating in the blood of ruminants (see Shaw, 1956).

Mammary tissue, which possesses high lipogenic activity (Balmain, Folley & Glascok, 1952) and which is able to oxidize acetoacetate (Terner, 1955), must synthesize acetoacetyl-coenzyme A as an intermediate; this may give rise to ketone bodies. As reported in previous papers (Terner, 1954, 1956b), the addition of fumarate in increasing amounts to respiring mammary homogenates metabolizing ¹⁴C-labelled acetate or pyruvate resulted in a progressive increase in the rate of fatty acid synthesis. In continuation of this work it was found that, when the rate of fatty acid formation was limited by decreasing the amount of added fumarate, acetoacetate was formed from acetate or pyruvate. In contrast with the powerful ketogenicity of pyruvate and acetate, glucose and lactate displayed only weak ketogenic activity. This analogous metabolic behaviour of glucose and lactate offers an explanation for the differences in ketogenic activity of the various substrates in terms of their ability to maintain an adequate level of reduced pyridine nucleotides in an actively oxidative environment and suggests a mechanism for the well-known antiketogenic properties of glucose. The results further indicate that the

lactating mammary gland may deserve consideration as a site of ketone-body production in dairy animals suffering from ketosis. A preliminary account of part of this work has been given (Terner, 1956c, 1957).

EXPERIMENTAL

The material, reaction mixtures, reagents and methods were the same as described in the earlier paper on lipogenesis in guinea-pig mammary homogenates (Terner, 1956b). Whole homogenates of mammary tissue were used; kidney-cortex homogenates were centrifuged at low speed for a few minutes to remove broken cells and nuclei.

Determination of acetoacetate. This was determined manometrically by the aniline method (Ostern, 1933; Edson, 1935) as modified by Krebs & Eggleston (1945). When radioactive substrates were employed, the 'bound CO₂' was first liberated from the medium by the addition of 1 ml. of a mixture of equal volumes of 50% citric acid and 3N-perchloric acid. The CO₂ was absorbed during the next 30 min. period in the NaOH in the centre wells of manometer flasks which had served to absorb the CO₂ during the measurements of O₂ consumption. The contents of the centre wells were transferred to a few millilitres of water in test tubes. The centre wells were washed with dil. HCl and charged with fresh portions (0.2 ml.) of 2N-NaOH and a strip of filter paper. The acetoacetate was then decarboxylated by adding 1 ml. of aniline citrate from the side arms of the vessels. After the reaction was complete, the contents of the centre wells were transferred to a second set of test tubes. Since aniline will also decarboxylate oxaloacetate, treatment with Al₂(SO₄)₃ (Krebs & Eggleston, 1945) was in some experiments interposed between the collection of the respiratory CO₂ and the addition of

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