Experiments on the Function of Pantothenate in Bacterial Metabolism

By G. M. HILLS (Medical Research Council whole-time worker), *From the Department of Bacterial Chemistry (Medical Research Council), the Bland-Sutton and Courtauld Institutes, Middlesex Hospital, London, and the University, Sheffield*

(*Received 30 January 1943*)

The purpose of this account is to record results obtained in an attempt to determine the function of pantothenate in metabolism. From the observation that pantothenate stimulated O_2-uptake and especially CO_2-output [Williams, Mosher & Rohrmann, 1936; Pratt & Williams, 1939], it was thought that this nutrient might be specifically concerned with the metabolism of α-ketonic acids, since these are an important source, perhaps the only source, of respiratory CO_2. An abstract of earlier results [Hills, 1941] showed that many other substrates may be affected. In order to determine whether this was due to an intermediary metabolite common to these substrates, experiments on interaction between substrates both aerobically and anaerobically have now been carried out, and the course of pyruvate breakdown studied. While the work was in progress an abstract of similar work, with findings differing in some details, was published by Berkman, Dorfman & Koser [1942] who concluded in a later paper [Dorfman, Berkman & Koser, 1942] that pantothenate is concerned with a metabolic step involving pyruvate or a derivative, most likely that of the conversion of pyruvate to acetate. At the same time Teague & Williams [1942] produced evidence that pantothenate was not concerned with glucose phosphorylation, glucose or hexosediphosphate fermentation, or pyruvate decarboxylation. Their test systems were pantothenate-deficient yeast extract and chick brain. Later Pilgrim, Axelrod & Elvehjem [1942] showed that diminished oxidation of pyruvate occurs in pantothenate-deficient rat liver. Although the present study has been confined to a single bacterial species, the results may therefore have wider implications.

METHODS

Organisms. On account of the simplicity of its nutritional requirements, *Proteus morganii* was chosen for this work. Since pantothenate must be supplied in the medium [Pelczar & Porter, 1940], cells deficient in pantothenate, or with sufficient for optimum metabolism, may be obtained at will by regulation of the pantothenate content of the medium. Four strains from the National Collection of Type Cultures, Nos. 1707, 2814A, 2818 and 5845, were found to have similar pantothenate requirements for growth, and a similar deficiency in O_2-uptake when grown with suboptimal amounts. The results quoted refer to strain 2818 except where otherwise stated.

Culture media. The organisms were usually grown on the 'complete' amino-acid medium described by Gladstone [1939], with the addition of 1 g. $(NH_4)_2SO_4$ and 1 g. NH_4Cl/l., and of nicotinamide ($10^{-5}M$ final) and synthetic Ca (+) pantothenate (Merck), which were essential nutrients. Good crops of pantothenate-deficient organisms (afterwards described simply as 'deficient' where no ambiguity might arise) were obtained with $2–4 \times 10^{-8}N$ pantothenate. Aneurin ($10^{-7}M$) and riboflavin ($2 \times 10^{-6}M$) were sometimes added to avoid partial deficiencies of these factors, but these non-essential additions appeared not to influence the results. Later it was found possible to dispense with all the amino-acids except cystine, which was probably required as a source of organic S [cf. Fildes & Richardson, 1937; Gladstone, 1937]. This NH_3-cystine basal medium was made up similarly to the NH_3 medium of Fildes [1940] with the addition of 25 mg. cystine in NaOH and 1·25 mg. nicotinamide/l. final bulk. Lactate was omitted and glucose added to the basal medium after sterilization. Pantothenate requirements were the same as for the amino-acid medium.

A basal medium consisting of 1 % acid-hydrolysed casein [McIlwain, 1939] has also been used with the addition of glucose, nicotinamide and pantothenate as required. The organisms were not so deficient as those reaped from the synthetic media, even though the pantothenate concentration was reduced to $10^{-8}M$, the minimum necessary to support adequate growth.

All media were dispensed in 100 ml. quantities in 250 ml. conical flasks. Glucose (1·25 ml. *M*) and pantothenate were added at the time of inoculation. Except where otherwise stated the amino-acid medium was used.

Inoculum. In the early stages, to avoid carrying over pantothenate, a small inoculum of *ca.* 100 organisms/ml. was used but later this proved unsatisfactory. Instead, the inoculum for 100 ml. culture was 1 ml. of an 18–24 hr. culture on the same medium with $10^{-6}M$ pantothenate, which was found to give more reproducible results than cells raked from an agar slope and suspended to give a similar opacity (*ca.* 10^8 cells/ml.). Subcultures were never made from deficient cultures, so as to avoid training the organism to dispense with an added supply of pantothenate.

The inoculated medium was incubated in air for 40–48 hr. with the small inoculum, or for 20–24 hr. with the large inoculum. The cells were collected on the centrifuge and washed once. The yield was *ca.* 10 mg. dry wt. of deficient cells/100 ml. medium, which was enough for 3–5 Warburg vessels in most experiments. Except where stated otherwise the large inoculum was used in the experiments quoted.

Measurement of metabolism. Standard manometric methods as described by Dixon [1934] were used. The cell suspension in distilled water was suitably buffered and added to a salt mixture in Warburg vessels, so that with any additions from the side-bulbs the final composition of the medium was that of Krebs & Henseleit [1932], with replacement of KH_2PO_4 by KCl, and limitation of the Ca^{++} content to $3 \times 10^{-5} N$ including the Ca pantothenate added to certain vessels. This excluded any possible effect on metabolism of the Ca^{++} in the pantothenate, and there were no difficulties due to precipitation when the $NaHCO_3$ buffer was replaced by 30 mM phosphate in the measurement of O_2-uptake by the direct method. Except where otherwise stated the pantothenate concentration was $3 \times 10^{-6} N$. In anaerobic experiments the last traces of O_2 were removed by yellow P in the centre well. The temperature was 37°.

Substrates. Pyruvate was purified by vacuum distillation according to Wendel [1932], and was stored as 5 M solution at 0–5°. It was determined by the carboxylase method according to the details of Westerkamp [1933].

Oxalacetic acid was prepared according to Wohl & Oesterlin [1901] and Wohl & Claussner [1907]. It was crystallized from hot acetone-benzene [Fenton & Jones, 1900] and determined, like acetoacetate, by the aniline citrate method [Edson, 1935; Greville, 1939]. I am indebted to Mr D. E. Hughes for pure preparations of pyruvic and oxalacetic acids. Both these substrates were neutralized just before use with the calculated quantity of $NaHCO_3$.

Except where otherwise stated the final concentration of all substrates was 224 μl. in 3·33 ml. per vessel, i.e. 3 mM (each form where *dl*-mixtures were used).

RESULTS

Pantothenate requirements for growth and metabolism

Table 1 shows the influence of different concentrations of pantothenate in the culture medium on O_2-uptake of the washed cells. The low metabolism of those grown with 1–4 $\times 10^{-8} M$ pantothenate was restored to normal by $3 \times 10^{-6} M$ pantothenate in the final suspension. If we take the yield of organisms into account, however, the amount, in deficient cells, of the enzyme requiring pantothenate was, even when metabolism had been activated subsequently by addition of pantothenate, only 55 % of that produced under optimal conditions. Table 2 shows that the concentration of pantothenate used in the final suspension was ample for optimal activation of both oxidation and fermentation of pyruvate.

Table 1. *Effect of pantothenate concentration during cultivation on O_2-uptake*

Cells grown from a small inoculum. 20 mM pyruvate added after shaking 1 hr. in air.

	Pantothenate (M) during growth		
	8×10^{-9}	4×10^{-8}	10^{-6}
Crop (mg. dry cells/100 ml. medium)	10	21	32
Q_{O_2} (μl./mg. cells):			
No pantothenate	17	16	55
With pantothenate	105	47	56
O_2-uptake (μl./100 ml. culture/hr.):			
No pantothenate	170	340	1760
With pantothenate	1050	990	1790

Table 2. *Effect of added pantothenate on aerobic and anaerobic metabolism of pyruvate by deficient cells*

Strain 2818 grown on hydrolysed casein with $2 \times 10^{-8} M$ pantothenate. The suspensions were shaken for 30 min. in the presence of the stated concentrations of pantothenate before adding pyruvate (20 mM). The data are for the period 40–70 min. after adding the substrate.

Pantothenate added M	Q_{O_2}	$Q^{N_2}_{acid+gas}$
0	54	17
10^{-9}	—	21
3×10^{-9}	59	32
10^{-8}	71	52
3×10^{-8}	81	73
10^{-7}	94	—
10^{-5}	97	73

Influence of pantothenate on oxygen uptake

Specificity of influence of pantothenate. The effect on O_2-uptake was probably specific for pantothenate, since aneurin, hydrolysed pantothenate and a partially purified preparation of cozymase were all ineffective (Table 3). The cozymase preparation stimulated the organism under appropriate conditions (cozymase-deficient cells grown on the hydrolysed casein medium with no added nicotinamide). Hence its ineffectiveness on pantothenate-deficient cells was not likely to be due to its inability to reach the active centres.

Table 3. *Specificity of influence of pantothenate on oxygen uptake of deficient cells*

Strain 2818 grown on pantothenate-deficient medium, i.e. hydrolysed casein with added pantothenate ($10^{-8} M$) and nicotinamide ($10^{-5} M$), and on nicotinamide-deficient medium, i.e. hydrolysed casein with added pantothenate ($10^{-6} M$) and no added nicotinamide.

Addition	Conc. M	Q_{O_2} (20–80 min.) Panto-thenate-deficient	Nicotin-amide-deficient
None	—	28	43
Pantothenate	$1{\cdot}5 \times 10^{-7}$	82	45
*Hydrolysed pantothenate	$1{\cdot}5 \times 10^{-6}$	34	—
Aneurin	3×10^{-5}	34	—
Cozymase	2×10^{-6}	35	80

* N HCl 10 min. at 100°.

Substrate specificity. Table 4 shows the effect of pantothenate on the O_2-uptake of deficient cells in the 1st hr. after adding a variety of substrates. The rate of O_2-uptake with pyruvate in the presence of pantothenate is taken as 100, as an arbitrary standard of comparison between different experiments. The total uptake after 4 hr. is given, expressed as mol./mol. substrate supplied. To obtain comparable results in different experiments, the cells from 200 ml. culture were always used for an experiment with 10 vessels. This procedure gave a greater uniformity than that in which a constant dry weight of organisms was used in each vessel. With the exception of glucose, for which the O_2-uptake was not greatly affected by the addition of pantothenate, and of succinate, pyruvate gave a more rapid rate of O_2-uptake than any other substrate tested.

Table 4. *Substrate specificity for influence of pantothenate on oxygen uptake of deficient cells*

To facilitate comparison of substrates with the same C skeleton but different degrees of oxidation, compounds are arranged in order of decreasing number of C atoms and increasing degree of oxidation for a given number of C atoms.

Substrate	Relative rate of O_2-uptake for 1st hr. (uptake with pyruvate and added pantothenate = 100)		Mol. O_2 taken up in 4 hr.		Theory for completion
	No pantothenate	With pantothenate	No pantothenate	With pantothenate	
(*a*) Using organisms grown from a large inoculum					
Citrate	9	38	0·07	0·38	4·5
dl-Glutamate	17	73	0·15	0·88	4·5*
α-Ketoglutarate	14	55	0·17	0·74	4·0
Succinate	36	104	0·33	1·24	3·5
Fumarate	28	88	0·22	1·06	3·0
l(−) Malate	38	58	0·27	0·63	3·0
dl-Aspartate	20	61	0·19	0·84	3·0*
Oxalacetate	39	80	0·35	1·13	2·5
Glycerol	35	64	1·13	2·14	3·5
d- or *l*-Lactate	33	86	0·24	0·97	3·0
dl-Alanine	17	41	0·15	0·39	3·0*
Pyruvate (10 exp.)	34	100 (Q_{O_2} = 75)	0·36	1·18	2·5
Standard deviation	9	(11)	0·13	0·25	—
None (11 exp.)	6	16	0·04	0·12	—
Standard deviation	3	6	0·03	0·04	—
(*b*) Using organisms grown from a small inoculum					
Glucose	150	166	1·51	2·00	6·0
Galactose	17	33	0·17	0·60	6·0
Butyrate	2	8	0·09	0·14	5·0
β-Hydroxybutyrate	8	14	0·08	0·17	4·5
Acetoacetate	11	14	0·08	0·14	4·0
Succinate	26	36	—	—	3·5
l-Malate	9	17	—	—	3·0
Propionate	4	5	0·06	0·10	3·5
dl-Glyceraldehyde	13	24	0·12	0·24	3·0*
dl-Lactate	31	74	0·37	1·18	3·0
Pyruvate (6 exp.)	39	100 (Q_{O_2} = 51)	0·54	0·86	2·5
Standard deviation	6	(8)	0·10	0·13	—
Ethanol	16	22	0·23	0·39	3·0
Acetaldehyde	7	10	0·19	0·31	2·5
Acetate	8	13	0·15	0·35	2·0
Formate	33	41	0·36	0·52	0·5
None (5 exp.)	7	14	0·07	0·14	—
Standard deviation	4	6	0·03	0·04	—

* Calculated on the assumption that only one optical isomer is oxidized.

At one stage in the work it was found that malate, and to a less extent succinate, gave slower O_2-uptake (Table 4 (*b*)) than was shown by Hills [1941], the lower rate being in agreement with the report of Berkman *et al.* [1942]. Attempts to restore the activity by the addition of animal ash or Mn^{++} to the culture medium [cf. Woolley, 1941], which was suspected to be suboptimal with regard to trace elements, were unsuccessful, but with larger inocula (Table 4 (*a*)) it was found possible to get better growth and an increase in the rate of O_2-uptake in the presence of the C_4-dicarboxylic acids without any special additions to the medium. Since this study had to be abandoned before it was found possible to repeat the work on 12 out of 24 substrates using organisms grown from larger inocula, the data are not all strictly comparable and are divided between two lists: for organisms grown from (*a*) large and (*b*) small inocula respectively. As will be seen later, the latter list probably includes only two substrates, acetate and formate, which may be of importance in the function of pantothenate.

Other substrates which promoted rapid O_2-uptake (50–100 % of the rate with pyruvate) in the presence of pantothenate were: glutamate, α-ketoglutarate, glycerol and both optical isomers of lactate. Galactose, citrate, glyceraldehyde, alanine and formate gave moderate rates of O_2-uptake in the presence of pantothenate, initially 20–50 % of that with pyruvate. With formate, the rate was independent of pantothenate in the first 15 min. but fell off rapidly, more so without added pantothenate. With acetaldehyde and acetate, the initial rate was similar to that with the blank, but was maintained. Ethanol gave a slightly greater rate, while butyrate, β-hydroxybutyrate, acetoacetate and propionate, with or without pantothenate, gave rates equal to those without added substrate.

Substrate competition. As the maintenance of constant rate of O_2-uptake was more important than an attempt to determine conditions for complete oxidation, the substrate concentration in these experiments was increased to 5 m*M* (Table 5). Under these conditions, although succinate promoted rapid O_2-uptake, the effect of pantothenate was relatively small, suggesting that the previous effect (Table 4) was due to oxidation of some of the products of succinate metabolism. The smaller effect of pantothenate was not due to inability of the suspension to take up O_2 at a greater rate, since succinate and pyruvate together gave a higher O_2-uptake in the

Table 5. *Effect of other substrates on oxygen uptake of deficient cells in presence of pyruvate and pantothenate*

Exp.	Substrate, 5 m*M* each optical component	Relative rate of O_2-uptake (rate with pyruvate and added pantothenate = 100): No pantothenate	With pantothenate	With pantothenate and pyruvate
1	Pyruvate	40	100	101*
	Succinate	121	133	176
	dl-Glutamate	17	49	106
2	Pyruvate	12	100	—
	Succinate	—	—	154
	α-Ketoglutarate	—	58	119
	Oxalacetate	—	109	105
	dl-Aspartate	—	35	117
3	Pyruvate	40	100	100*
	Glucose	167	187	188

* Double pyruvate concentration.

presence of pantothenate. The bulk of the O_2-uptake with succinate therefore did not go through the same system as that with pyruvate, but the small part increased by pantothenate may have done so. With glutamate, on the other hand, the maximum rate of O_2-uptake in the presence of pyruvate was little more than that with pyruvate alone, suggesting that a common system was involved. α-Ketoglutarate and aspartate behaved somewhat similarly to glutamate, while oxalacetate, as might be expected, showed almost the same uptake with or without pyruvate. No conclusion as to the role of pyruvate in glucose oxidation can be drawn, since the failure of pyruvate to increase the uptake in the presence of glucose can have no significance in the absence of evidence to show that substrate activation was the limiting factor in the chain of oxidation.

Influence of pantothenate on anaerobic metabolism

Of the nine substrates tested, all of which gave rapid O_2-uptake, only glucose, pyruvate and oxalacetate showed anaerobic fermentation detectable manometrically in $NaHCO_3$ buffer (Table 6). The fermentation of glucose, though more rapid than that of the other substrates, was of little interest from the point of view of the function of pantothenate, since it was little influenced by this substance. A slight decrease in the rate of gas production with glucose was observed in the presence of pantothenate (presumably due to diversion of pyruvate from the oxido-reduction with triosephosphate in the Embden-Meyerhof-Parnas scheme). The reaction was complete after the evolution of nearly 2 mol. gas calculated as CO_2. The rate was uninfluenced by excess fumarate except for the continued slow evolution of gas in the presence of pantothenate after the process without fumarate had ceased. The anaerobic metabolism of pyruvate, which was already known to be markedly dependent on pantothenate (Table 2) gave about 0·75 mol. gas (0·66–0·86 in 12 experiments) calculated as CO_2,

Table 6. *Effect of added pantothenate on anaerobic metabolism of deficient cells*

Relative production of acid + gas (calculated as CO_2) from various substrates in 1st hr.: pyruvate with added pantothenate = 100. In parentheses is given mol. gas produced/mol. substrate at the time of the completion of the reaction in the presence of pantothenate. Fumarate, where present, *ca.* 3700 μl., i.e. 50 m*M*.

Substrate	With substrate alone: No pantothenate	With pantothenate	With substrate + fumarate: No pantothenate	With pantothenate	With substrate + pyruvate: No pantothenate	With pantothenate
None	0	−8	8	8	15 (0·13)	100 (0·76)
Pyruvate	15 (0·13)	100 (0·76)	50 (0·44)	286 (0·95)	—	—
Glucose	900 (1·90)	820 (1·97)	935 (1·88)	910 (2·20)	—	—
dl-Glutamate	−18	−19	—	—	25 (0·11)	154 (0·76)
α-Ketoglutarate	0	6	19	36	73	145
Succinate	−2	−20	—	—	43 (0·20)	152 (0·74)
l(−) Malate	−3	−3	—	—	64 (0·29)	300 (0·81)
dl-Aspartate	3	1	−4	12	118 (0·75)	267 (0·84)
Oxalacetate	14	59	29	73	—	—
Glycerol	4	4	17	26	—	156

suggesting that two reactions already recognized in bacterial fermentation may be taking place:

(1) $2CH_3.CO.COO^- + H_2O = CH_3.CHOH.COO^- + CH_3.COO^- + CO_2$

as in the cocci [Krebs, 1937*a*] and *Esch. coli* at an acid *p*H [Krebs, 1937*b*];

(2) $CH_3.CO.COO^- + HCO_3^- = CH_3.COO^- + HCOO^- + CO_2$

as in aerobically grown *Esch. coli* [Krebs, 1937*b*] and in streptococci [Barron & Lyman, 1939].

In the presence of excess fumarate the rate was increased nearly threefold and the yield of gas became 1 mol./mol. pyruvate. This may be explained by assuming that fumarate replaced that part of the pyruvate acting as H-acceptor as in the oxido-reduction of reaction (1). Oxalacetate gave initial rates which were less than with pyruvate, and which fell off so that it was impossible to observe the completion of the reaction. The acceleration produced by fumarate in this case was also small. α-Ketoglutarate and glycerol stimulated no evolution of gas when present as sole substrates, but gave a small amount in the presence of fumarate. Glutamate, succinate, fumarate, malate and aspartate showed no evolution of gas, while aspartate did not produce gas even in the presence of fumarate. All these substances increased the rate of gas production in the presence of pyruvate by at least 50%—in the case of the C_4-dicarboxylic acids (excepting succinate) by 100–200%—without significantly altering the total amount of gas produced. Oxalacetate gave a similar increase in rate with pyruvate which was studied in more detail (Table 7). The reaction with 112 μl. pyruvate was complete in 2 hr. when 75 μl. (0·69 mol.) gas had been evolved in excess of the blank. With 224 μl. pyruvate the rate was little increased and the reaction was not quite complete in 4 hr., but with 112 μl. pyruvate and 112 μl. oxalacetate the reaction was complete in 1 hr., the initial rate having been doubled. The yield was 1 mol. gas/mol. pyruvate. Even in the absence of pantothenate this reaction was complete in 3 hr. Exp. 2 (Table 7) showed that 0·25 mol. oxalacetate was sufficient to double the rate of fermentation, while a small effect was shown with 0·05 mol. Exp. 3 (Table 7) showed that the effect was truly catalytic, since even if the blank values were deducted (and this is not always justifiable [Krebs, 1937*b*]), the increase in output of gas due to 22 μl. (0·10 mol.) oxalacetate was 26 and 37 μl. in 1 and 2 hr. respectively, while the oxalacetate alone produced less than half these amounts. Although oxalacetate increased the rate of fermentation of pyruvate even in the absence of added pantothenate, the effect was not so great, and was possibly due to residual pantothenate in the cells. In contrast, the effects of aneurin and oxalacetate on pyruvate dismutation by aneurin-deficient staphylococci were identical either singly or together [Smyth, 1940]. Smyth suggested that the function of aneurin was the carboxylation of pyruvate to form oxalacetate, the latter acting as a catalyst in the dismutation (cf. Krebs & Eggleston [1940] for pigeon liver). In the present work, Exp. 1 (Table 7) did suggest, in fact, that an equimolecular amount of oxalacetate excluded pyruvate as a H-acceptor (thus increasing the yield of gas to 1 mol./mol. pyruvate) and that this reaction was catalysed by pantothenate.

Table 7. *Catalysis of anaerobic metabolism in the presence of pyruvate by oxalacetate with or without added pantothenate*

	Substrates		μl. acid + gas produced in periods					
			0–1 hr.		0–2 hr.		0–3 hr.	
Exp.	Pyruvate μl.	Oxalacetate μl.	No pantothenate	With pantothenate	No pantothenate	With pantothenate	No pantothenate	With pantothenate
1	0	0	−11	−11	−11	−10	−7	−5
	112	0	1	44	12	65	19	72
	224	0	7	51	16	100	26	134
	0	112	16	11	27	22	33	26
	112	112	41	100	70	102	104	105
2	0	0	10	6	15	10	19	10
	224	0	15	34	26	66	35	99
	213	11	20	38	31	76	42	115
	168	56	25	60	42	108	57	145
3	0	0	9	2	14	6	20	12
	202	0	24	47	40	105	56	147
	0	22	13	12	18	15	23	21
	202	22	41	83	64	151	77	165

Aerobic and anaerobic metabolism of pyruvate and the influence of pantothenate

Optimum p*H*. Table 8 shows that the optimum *p*H for pyruvate oxidation, with or without pantothenate, was about 5·9, but the optimum effect of

Table 8. *Optimum pH for influence of pantothenate on O_2-uptake with pyruvate as substrate*

(Initial *p*H calculated from composition of Na phosphate buffers freshly prepared in CO_2-free water.)

	Q_{O_2} 30–75 min.	
Initial *p*H	No pantothenate	With pantothenate
ca. 4·6	31	39
5·9	37	66
6·6	28	61
7·0	20	58
7·3	16	47
7·8	11	40

pantothenate occurred at about *p*H 7. Above this *p*H the proportion of O_2-uptake due to added pantothenate was almost constant at 70% of the total, suggesting that catalysis by pantothenate was being limited by the rate of formation of some intermediate, e.g. perhaps oxalacetate. Attempts to determine the optimum *p*H for fermentation by variation of the concentration of $NaHCO_3$ (Table 9)

Table 9. *Optimum pH for influence of pantothenate on anaerobic metabolism of pyruvate*

(Initial *p*H calculated from composition of $NaHCO_3$ buffer in equilibrium with 5·7% CO_2 in N_2.)

		μl. acid+gas			
		0–45 min. 1·5 m*M* pyruvate in *ca.* 150 m*M* salts		60–120 min. 3 m*M* pyruvate in *ca.* 280 m*M* salts	
$NaHCO_3$ m*M*	Initial *p*H	No pantothenate	With pantothenate	No pantothenate	With pantothenate
1	*ca.* 6·0	0	27	—	—
3	*ca.* 6·5	10	24	20	26
10	7·0	14	46	14	24
30	7·5	19	52	17	36
90	8·0	17	49	20	45
270	8·5	—	—	18	38

showed that it was not less than 7·5–8·0 at the salt concentrations normally used in this work. When the $NaHCO_3$ concentration was increased to 0·27 *M* to give *p*H 8·5 in equilibrium with 5·7% CO_2 (NaCl being added to other vessels to maintain the same total salt concentration) the usual lag before fermentation reached its maximum rate was prolonged for at least ½ hr., but in the 2nd hour the optimum *p*H both for total fermentation and for the increase due to added pantothenate was 8·0. Under these conditions almost exactly 1 mol. gas was produced at the optimum, while at *p*H 8·5 little more than half as much was obtained, and at *p*H 7·0 little more than one-third. These figures must be accepted with caution, however, since cessation of fermentation was not shown to be due to exhaustion of substrate, and injury of the enzyme system at slightly unfavourable *p*H values by the high salt concentration was not excluded. At the lower salt concentration, 1 mol. gas was also obtained at *p*H 8·0 however, suggesting that under optimum conditions, reaction (2) occurred to the exclusion of the oxido-reduction (1).

Aerobic disappearance of pyruvate. Table 10 shows that without added pantothenate almost exactly 0·5 mol. O_2 was taken up during the period over

Table 10. *Relationship between pyruvate disappearance and O_2-uptake with and without added pantothenate*

(Strain 1707—grown on NH_3-cystine medium.)

	μl. reactant per vessel			
	No pantothenate		With pantothenate	
Time hr.	O_2-uptake	Pyruvate present	O_2-uptake	Pyruvate present
0	—	184	—	165
1	− 46	96	−108	9
2	− 93	3	−174	3
3	−113	4	−222	0

which the pyruvate disappeared, after which the O_2-uptake continued more slowly. With added pantothenate the rate of disappearance of pyruvate was at least doubled, the rate of O_2-uptake did not fall so much and had not fallen to zero when 1 mol. had been taken up. This experiment was carried out in phosphate buffer. With repetition of the experiment (Table 11) further information was obtained by determining the disappearance of acid

Table 11. *Relationship between pyruvate disappearance, R.Q. and acid disappearance, with and without added pantothenate*

(Bacteria from 300 ml. amino-acid medium in 5 vessels. 1·67 ml. suspending fluid per vessel. 20 min. at 37° to reach equilibrium with 5·7% CO_2 in air before making observations.)

	μl. reactant per vessel				
	No pantothenate		10^{-5} *M* pantothenate		
	Initially	Change after 4 hr.	Initially	Change after 2 hr.	Change after 4 hr.
Pyruvate	481	−465	431	−418	−423
Bound CO_2	813	+ 75	832	+152	+216
Total CO_2	832	+572	879	+641	+769
O_2	—	−307	—	−397	−525
R.Q.		1·86		1·61	1·00

\+ and − signs indicate appearance and disappearance respectively.

and formation of CO_2 by the method of Dickens & Šimer [1931] with $NaHCO_3$ as buffer. Without added pantothenate the average R.Q. over a 4 hr. period, during which all the pyruvate disappeared, approached the value 2·00, i.e. that for oxidation to acetate, though some further oxidation did occur.

With pantothenate the R.Q. was lower, even in the first 2 hr. (during which all the pyruvate disappeared), while in the second 2 hr. the data correspond to complete oxidation of some of the acetate presumably formed earlier. Unfortunately, the only evidence for the effect of pantothenate on pyruvate disappearance was that a smaller amount remained at the end of equilibration.

DISCUSSION

The evidence produced shows that the effect of pantothenate on metabolism was not confined to the oxidation of pyruvate to acetate, since a definite, though smaller, effect on O_2-uptake was seen after no measurable amount of pyruvate remained (Table 10), and possible degradation products of pyruvate such as acetate or formate themselves gave an effect on O_2-uptake (Table 4). Dorfman *et al.* [1942] concluded that the action of pantothenate may not be confined to that on pyruvate oxidation, though the bulk of their evidence was in favour of the idea that pantothenate acts on the conversion of pyruvate to acetate. It is a matter for future work to decide if the effect on O_2-uptake in the presence of acetate or formate is indirect. From this point of view the study of formate oxidation might be especially interesting since it promoted moderate O_2-uptake which was initially independent of pantothenate. In this connexion the experiments of Krebs [1937*b*] on the interaction of formate and fumarate in *Esch. coli* might be relevant. An indirect action, linked with the pyruvate system, did seem likely with the C_4 and C_5 dicarboxylic acids, on account of their effects both on O_2-uptake in the presence of pyruvate (Table 5) and on the fermentation of pyruvate (Table 6).

Whatever the role of pyruvate, however, in the oxidation of other substrates, quantitatively it was certainly the most important substrate influenced by pantothenate. Other vitamins are known to be concerned in pyruvate metabolism: (*a*) *Aneurin* as its pyrophosphate is concerned ultimately in pyruvate breakdown, though so far there is no agreement as to its precise function [cf. Barron, Lipton & Goldinger, 1941; Krebs & Eggleston, 1940; Lipmann, 1941; Quastel & Webley, 1941; Smyth, 1940]. (*b*) *Nicotinic acid* as diphosphopyridine nucleotide is concerned in the equilibrium between pyruvate and lactate, and also with that between oxalacetate and malate which, according to the views of Krebs & Eggleston [1940] and Smyth [1940], may be involved in pyruvate metabolism. (*c*) *Riboflavin* as riboflavin-adenine dinucleotide may be concerned in the later stages of H-transport, as in *Lactobacillus delbrückii* [Lipmann, 1939], though not directly concerned in substrate activation. Pantothenate should now be added to these as a component of pyruvate oxidase, though it has not been shown to play a part in the cell-free preparations from *Lactobacillus delbrückii* [Lipmann, 1939] and *Esch. coli* [Still, 1941]. Its participation, however, was not excluded since it was not shown that the preparations were free from pantothenate, and it is likely that the treatment was not drastic enough to release this factor from any protein with which it may have been bound. Pantothenate is known to be bound in liver, one of the richest natural sources, and is freed by autolysis [Williams, 1941; Williams, Truesdail, Weinstock, Rohrmann, Lyman & McBurney, 1938], but there was no autolytic process in the preparation of the enzyme of Lipmann or that of Still.

SUMMARY

1. In washed suspensions of *Proteus morganii* grown with suboptimal supplies of pantothenate, the addition of pantothenate increased the aerobic metabolism of pyruvate to a greater extent than that of 15 other substrates and the anaerobic metabolism of pyruvate more than that of nine other substrates tested.

2. The substrates which gave increases in O_2-uptake approaching that with pyruvate were six C_4 and C_5 dicarboxylic acids, and lactate. Little or no fermentation of these substrates, or of malate or glycerol, was detected manometrically, but all (except lactate, which was not tested) were shown to increase the rate of fermentation of pyruvate. With the C_4 dicarboxylic acids, including malate but not succinate, the increase was several-fold. With glutamate, α-ketoglutarate, aspartate or oxalacetate (and possibly succinate) as substrate, the increase in the O_2-uptake promoted by pantothenate tended to occur at the expense of that similarly promoted with pyruvate as substrate.

3. With glucose as substrate, fermentation was practically unaffected by pantothenate, and O_2-uptake was increased only in the later stages.

I wish to thank Dr P. Fildes who suggested this work, Prof. G. A. Clark for the provision of laboratory facilities at Sheffield, kindly arranged through Dr H. A. Krebs, and Prof. E. C. Dodds for the loan of apparatus and for laboratory facilities at the Courtauld Institute of the Middlesex Hospital. The continuation of the work to its present stage owes much to the suggestions, encouragement and criticism of Dr H. A. Krebs and Dr H. McIlwain.

REFERENCES

Barron, E. S. G. & Lyman, C. M. [1939]. *J. biol. Chem.* **127**, 143.
—— Lipton, M. A. & Goldinger, J. M. [1941]. *J. biol. Chem.* **141**, 957.
Berkman, S., Dorfman, A. & Koser, S. A. [1942]. *J. Bact.* **43**, 6.
Dickens, F. & Šimer, F. [1931]. *Biochem. J.* **25**, 973.
Dixon, M. [1934]. Manometric Methods. Cambridge: University Press.
Dorfman, A., Berkman, S. & Koser, S. A. [1942]. *J. biol. Chem.* **144**, 393.
Edson, N. L. [1935]. *Biochem. J.* **29**, 2082.
Fenton, H. J. H. & Jones, H. O. [1900]. *J. chem. Soc.* **77**, 77.
Fildes, P. [1940]. *Brit. J. exp. Path.* **21**, 67.
—— & Richardson, G. M. [1937]. *Brit. J. exp. Path.* **18**, 292.
Gladstone, G. P. [1937]. *Brit. J. exp. Path.* **18**, 322.
—— [1939]. *Brit. J. exp. Path.* **20**, 189.
Greville, G. D. [1939]. *Biochem. J.* **33**, 718.
Hills, G. M. [1941]. *Chem. Ind.* **60**, 220.
Krebs, H. A. [1937*a*]. *Biochem. J.* **31**, 661.
—— [1937*b*]. *Biochem. J.* **31**, 2095.
—— & Eggleston, L. V. [1940]. *Biochem. J.* **34**, 1383.
—— & Henseleit, A. [1932]. *Hoppe-Seyl. Z.* **210**, 33.
Lipmann, F. [1939]. *Nature, Lond.*, **143**, 436.
Lipmann, F. [1941]. *Advances in Enzymology*, **1**, 99.
McIlwain, H. [1939]. *Brit. J. exp. Path.* **20**, 330.
Pelczar, M. J. (Jr.) & Porter, J. R. [1940]. *Proc. Soc. exp. Biol., N.Y.*, **43**, 151.
Pilgrim, F. J., Axelrod, A. E. & Elvehjem, C. A. [1942]. *J. biol. Chem.* **145**, 237.
Pratt, E. F. & Williams, R. J. [1939]. *J. gen. Physiol.* **22**, 637.
Quastel, J. H. & Webley, D. M. [1941]. *Biochem. J.* **35**, 192.
Smyth, D. H. [1940]. *Biochem. J.* **34**, 1598.
Still, J. L. [1941]. *Biochem. J.* **35**, 380.
Teague, P. C. & Williams, R. J. [1942]. *J. gen. Physiol.* **25**, 777.
Wendel, W. B. [1932]. *J. biol. Chem.* **94**, 717.
Westerkamp, H. [1933]. *Biochem. Z.* **263**, 239.
Williams, R. J. [1941]. *Enzymologia*, **9**, 387.
—— Mosher, W. A. & Rohrmann, E. [1936]. *Biochem. J.* **30**, 2036.
—— Truesdail, J. H., Weinstock, H. H. (Jr.), Rohrmann, E., Lyman, C. M. & McBurney, C. H. [1938]. *J. Amer. chem. Soc.* **60**, 2719.
Wohl, A. & Claussner, P. [1907]. *Ber. dtsch. chem. Ges.* **40**, 2308.
—— & Oesterlin, C. [1901]. *Ber. dtsch. chem. Ges.* **34**, 1139.
Woolley, D. W. [1941]. *J. biol. Chem.* **140**, 311.

Micro-method for Estimating Vitamin A by the Carr-Price Reaction

By H. HOCH (Freedom Research Scholar), *From the Haymeads Emergency Hospital, Bishop's Stortford, and the Hale Clinical Laboratory, London Hospital*

(*Received 16 March 1943*)

In the micro-determination here described an attempt has been made to eliminate errors due to fading, differences in the colour of test and standard, and interference by non-specific absorption. The tube containing the reaction mixture is photographed in light of a selected wave-length range together with a series of standard tubes of equal dimensions (see Fig. 1), and the photographic film is analysed by means of photoelectric photometry. The advantages of this method are: (1) the working range is between 0·06 and 0·4 i.u. vitamin A in a sample; such small amounts cannot be determined by photoelectric colorimetry in the usual way, but it is necessary to work in this range when making serial investigations on the blood of small animals and is convenient in investigations on human beings; (2) rapid mixing is ensured as a consequence of the small volume of the reacting liquids (0·02 ml.); (3) a record of the colour produced can be obtained as early as 2 sec. after the initiation of the reaction, and its course can be followed photographically with accurate timing, thus avoiding the lag of galvanometer readings in photoelectric methods [Dann & Evelyn, 1938; Yudkin, 1941]; (4) the readings are independent of fluctuations in the light source during the reaction.

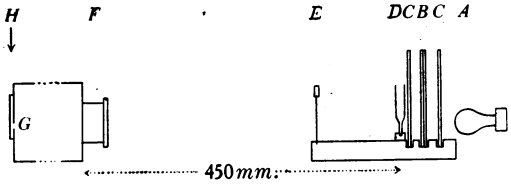

Fig. 1. Photographic arrangement. *A*, light source, two 100 W. coiled coil bulbs. *B*, space for filters: (1) 'Calorex' 2 mm.; (2) 'Signal green' 1 mm. (manuf. by Messrs Chance, 10 Princes St, S.W. 1). *C*, diffusing glass 2 mm. *D*, reaction tube and standard tubes. *E*, Wratten 26 red gelatin filter. *F*, enlarging lens at suitable distance to give image of tubes of about natural size. *G*, slit, 3·5 mm. wide, in front of slide which is moved in vertical direction. *H*, slide.