SUMMARY

1. It is shown that free haematins will react with nitric oxide under certain conditions giving compounds with characteristic absorption spectra.

2. The reactions of nitric oxide with both protohaematin and urohaematin were examined. In acid solution each of these haematins changes colour from reddish brown to a bright red in the presence of nitric oxide, and two strong absorption bands appear in the green region of the spectrum while the Soret band of the haematin is shifted to a longer wavelength.

3. Urohaematin in alkaline solution reacts similarly with nitric oxide and can be reduced to give a NO-urohaem compound with a single broad absorption band in the visible region. The results obtained with alkaline protohaematin are less clear-cut.

4. The absorption spectra of the NO-compounds of proto- and uro-haematin under various conditions have been determined.

5. No spectroscopic evidence was obtained for any reaction between fluoride, azide or sulphide with free haematins, although these substances all form well-defined compounds with methaemoglobin and other haemoproteins.

6. The reactions of different substances with free iron-porphyrins were compared with those given with haemoproteins. Of all the substances examined only nitric oxide and the cyanide ion are capable of co-ordinating with the bivalent and trivalent iron of haem and haematin respectively, both when they are free and when they form the prosthetic groups of haemoproteins.

I wish to thank Dr R. Hill, F.R.S., and Dr E. F. Hartree for their helpful suggestions and interest in this work.

REFERENCES

- Anson, M. L. & Mirsky, A. E. (1928). *J. gen. Physiol.* **12**, 273.
 Balthazar, V. & Philippe, M. (1926). *Ann. Méd. lég.* **6**, 137.
 Bechtold, E. (1935). *Der Muskelfarbstoff*. Stuttgart.
 Brewster, D. (1834). *Trans. Roy. Soc. Edinb.* **12**, 519.
 Coryell, C. D., Pauling, L. & Dodson, R. W. (1939). *J. phys. Chem.* **43**, 825.
 Davies, T. H. (1940). *J. biol. Chem.* **135**, 597.
 Drabkin, D. L. & Austin, J. H. (1935). *J. biol. Chem.* **112**, 51.
 Fischer, H. & Orth, H. (1934). *Die Chemie des Pyrrole*, **2**. Leipzig: Akademische Verlagsgesellschaft.
 Gamgee, A. (1898). *Textbook of Physiology*, ed. E. A. Schäfer, vol. 1, p. 258. Edinburgh and London: Young, J. Pentland.
 Gonella, A. & Vannotti, A. (1943). *Z. ges. exp. Med.* **112**, 405.
 Hartree, E. F. & Harpley, C. H. (1949). *Biochem. J.* **44**, 637.
 Haurowitz, F. (1924). *Hoppe-Seyl. Z.* **137**, 1.
 Haurowitz, F. (1926). *Hoppe-Seyl. Z.* **151**, 130.
 Haurowitz, F. (1951). *J. biol. Chem.* **193**, 443.
 Haurowitz, F. & Waelsch, H. (1929). *Hoppe-Seyl. Z.* **182**, 82.
 Hermann, L. (1865). *Arch. Anat. Physiol., Lpz.*, p. 469.
 Hill, R. (1929). *Proc. Roy. Soc. B*, **105**, 112.
 Hill, R. & Holden, H. F. (1926). *Biochem. J.* **20**, 1326.
 Keilin, D. & Hartree, E. F. (1937). *Nature, Lond.*, **139**, 548.
 Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
 Keilin, J. (1949a). *Biochem. J.* **45**, 440.
 Keilin, J. (1949b). *Biochem. J.* **45**, 448.
 Keilin, J. (1950). *Nature, Lond.*, **165**, 151.
 Keilin, J. (1952). *Biochem. J.* **51**, 443.
 Kiese, M. & Kaeske, H. (1942). *Biochem. Z.* **312**, 121.
 Linossier, G. (1887). *C.R. Acad. Sci., Paris*, **104**, 1296.
 Schalfjeff, M. (1885). *J. Soc. phys.-chim. russe*, p. 30; from *Ber. dtsch. chem. Ges.* **18** (Referat Bd.), 232.
 Schumm, O. (1925). *Hoppe-Seyl. Z.* **149**, 111.
 Stitt, F. & Coryell, C. D. (1939). *J. Amer. chem. Soc.* **61**, 1263.
 Warburg, O., Negelein, E. & Christian, W. (1929). *Biochem. Z.* **214**, 26.

Glutamine Synthesis by *Micrococcus pyogenes* var. *aureus*

By B. A. FRY

Medical Research Council Unit for Chemical Microbiology, School of Biochemistry, University of Cambridge*

(Received 8 October 1954)

The synthesis of glutamine has been studied by Elliott (1948, 1951) and Speck (1949) in cell-free extracts of sheep brain and pigeon liver. Synthesis of glutamine occurred when the enzyme preparation was incubated with ammonia, magnesium ions, L-glutamic acid and adenosine triphosphate (ATP). Replacement of ammonia by hydroxylamine led to the synthesis of a hydroxamic acid, which by analogy with glutamine was presumably γ -glutamyl-

* Present address: Department of Microbiology, University of Sheffield.

hydroxamic acid. Since hydroxamic acids can be readily detected and estimated by the formation of a coloured complex in the presence of ferric ions at an acid pH (Lipmann & Tuttle, 1945), synthesis of glutamylhydroxamic acid is a convenient test for detecting the ability of an enzyme preparation to synthesize glutamine. Concurrent with Elliott's studies of the sheep-brain system, Elliott & Gale (1948) showed that cell-free extracts of *Micrococcus pyogenes* var. *aureus* synthesized glutamylhydroxamic acid. The work now described presents

a more detailed study of glutamine synthesis by this organism and the results of an investigation of the distribution of the enzyme system in various bacteria. A preliminary account of this work has been given previously (Fry, 1949).

MATERIALS AND METHODS

Organisms, growth media and preparation of washed cell suspensions. Unless stated to the contrary, the organism used was *Micrococcus pyogenes* var. *aureus* strain Duncan (Medical Research Council Unit for Chemical Microbiology, University of Cambridge). The organisms used in the distribution survey were *Bacterium cadaveris* National Collection of Type Cultures (N.C.T.C.), no. 6578, *Clostridium welchii* SR12 (N.C.T.C. no. 6784), *Escherichia coli* K4 (Department of Biochemistry, Sheffield University) and *Esch. coli*, N.C.T.C. no. 86, *Lactobacillus arabinosus* 17-5 (Department of Biochemistry, Sheffield University), *Lactobacillus casei* National Collection of Industrial Bacteria no. 8019, *Leuconostoc mesenteroides* American Collection of Type Cultures no. P60, three strains of *Micrococcus pyogenes* var. *aureus* N.C.T.C. nos. 563, 1560 and 6773, *Proteus vulgaris* (Medical Research Council Unit for Chemical Microbiology, Cambridge), two strains of *Streptococcus faecalis* N.C.T.C. nos. 6782 and 6783. *Streptococcus haemolyticus* strain Richards N.C.T.C. no. 5631 was used in the microbiological assay of glutamine. The growth medium, initial pH 7.0, contained 3% (v/v) enzymic (trypsin or papain) digest of casein (i.e. the equivalent of 3 g. digested casein/100 ml.) and 0.1% (w/v) Marmite, together with glucose, 2% (w/v) for coliform organisms and 1% for other bacteria. The medium was dispensed in flasks for lactobacilli and clostridia, and in Roux bottles (150 ml./bottle) for other organisms. Except for *Cl. welchii*, organisms were subcultured at 37° in test tubes containing 5 ml. of the same medium; bulk cultures were inoculated with 0.5 ml. of a 16 hr. subculture/150 ml. medium, and incubated at 37° for 6 hr. *Cl. welchii* was subcultured in Robertson's cooked meat medium; bulk cultures were inoculated with 4 ml. of the broth from a 16 hr. subculture/litre of freshly autoclaved medium and incubated anaerobically at 37° for 6 hr. The organisms were harvested by centrifuging, washed once with distilled water and then suspended in distilled water: in the case of *M. pyogenes*, the suspension was adjusted to 30 mg. dry wt./ml.

Dry-weight determinations. These were made turbidometrically using the Hilger Spekker absorptiometer with neutral grey filters. A standard curve relating optical density and turbidity was prepared for each organism using a suspension of known dry weight. The dry weights of the standard suspensions were determined by weighing after the suspensions had been dried at 105° for 4 hr.

Preparation of cell-free extracts. Suspensions were shaken for 30 min. with glass beads (Chance Bros, Ballotini no. 14), 10 g./tube, in a Mickle disintegrator (Mickle, 1948). After removing the beads by filtration, the filtrate was centrifuged at 1000 g for 30 min. The clear yellowish cell-free supernatant fluid was decanted from the cell debris and is referred to as the 'enzyme preparation (Mickle)'.

Preparation of acetone powders of M. pyogenes. The washed cell suspension was treated with 5 vol. of acetone, previously cooled to 4°, and allowed to stand for 15 min. in an ice bath. The insoluble material was collected by filtration and washed

successively with acetone, acetone-ether (1:1, by vol.) and finally, ether (all previously cooled to 4°).

Chemicals. Hydroxylamine hydrochloride, cysteine hydrochloride and L-glutamic acid (L. Light and Co. Ltd., Colnbrook, Bucks) were recrystallized as their hydrochlorides. ATP was prepared from rabbit muscle (LePage, 1949) as the monobarium salt (Bailey, 1949) and solutions of the sodium salt (Bailey, 1942) were stored at -10° (Bailey, 1949). Aerosol OT was supplied by the Hercules Powder Co., Wilmington, U.S.A.

The following substances were prepared according to the methods in the appropriate reference: glutathione (Pirie, 1930), glutamine (Vickery, Pucher & Clark, 1935; purified by the method of Hughes, 1950), γ -glutamylhydroxamic acid, methionine sulfoxide and methionine sulphone (Roper & McIlwain, 1948).

The glyoxaline buffers (Kirby & Neuberger, 1938) were prepared from the appropriate base hydrochlorides (British Drug Houses Ltd., London) and NaOH.

Analytical procedures

Glutamylhydroxamic acid. This was determined colorimetrically (Lipmann & Tuttle, 1945) using synthetic glutamylhydroxamic acid as the standard. In this procedure the final pH affects the intensity of the coloured ferrihydroxamic acid complex and it was found that the conditions for maximal colour development for glutamylhydroxamic acid were the same as those described for acetylhydroxamic acid.

Phosphate. This was determined colorimetrically by the method of Fiske & Subbarow (1925). ATP concentrations are based on the amount of acid-labile P (P_{10}) liberated by hydrolysis in N-HCl at 100° for 10 min. (Bailey, 1949).

Total nitrogen. This was determined by the method of Chibnall, Rees & Williams (1943). Ammonia was estimated by trapping in boric acid containing a mixed indicator (Conway & O'Malley, 1942) and titrating with standard HCl from a micro-burette.

Glutamine was estimated using the Parnas apparatus (Parnas & Heller, 1924). In the experiments dealing with glutamine synthesis, the reaction was stopped by the addition of trichloroacetic acid (1 ml. 40% (w/v)/4.5 ml. experimental fluid) and after the precipitated protein had been removed by centrifuging, a sample (4 ml.) of the supernatant was brought to pH 7 with N-NaOH and stored at -10° until analysed. Each sample was transferred quantitatively to the weighed flask of the Parnas apparatus, 2 ml. 20% (w/v) KBO_3 was added and the free NH_3 removed by three distillations, in each of which 10 ml. of distillate were collected. After the third distillation, when the NH_3 in the distillate was equal to that in the apparatus blank, the interior of the apparatus above the flask was carefully washed with NH_3 -free water and the flask detached and weighed. The weight of the sample plus the various washings could thus be found and after conc. H_2SO_4 had been added to a final concn. of 5%, the flask was placed in a boiling-water bath for 10 min. Under these conditions the amide group of glutamine is hydrolysed (Krebs, 1935). After cooling the flask in ice its contents were brought to pH 10.5 by the addition of 15N-NaOH, and the NH_3 released by the acid hydrolysis was distilled and estimated.

A Hilger Spekker absorptiometer was used in all the colorimetric procedures, with a red filter (Ilford no. 608, peak transmission at 680 $m\mu$.) for phosphate determina-

Table 1. *Synthesis of glutamylhydroxamic acid by whole cells and by preparations from disintegrated cells*

Complete system contained 1 ml. enzyme preparation, 0.04M sodium acetate buffer pH 5.4, 0.44M-NH₂OH, 0.04M-MgCl₂, 0.05M sodium glutamate, 0.006M ATP; total vol., 4.5 ml. Incubated 1 hr. at 37°. Disintegrated cells centrifuged to give cell-free supernatant and insoluble particles.

Enzyme preparation	Hydroxamic acid (μ moles/4.5 ml.)				
	Complete system	No ATP	No MgCl ₂	No glutamate	Boiled enzyme
Washed cell suspension (30 mg. dry wt./ml.)	0.2	0.0	0.0	0.0	0.0
Supernatant	1.5	0.0	0.2	0.0	0.0
Suspension of insoluble particles	0.3	0.1	0.1	0.2	0.0

tions, and green (no. 604, peak transmission at 520 m μ) for hydroxamic acid.

Experimental systems were incubated in open test tubes in a water bath at 37° and ATP was added after they had been in the bath for 10 min.

Standard experimental systems

After the preliminary experiments, the standard system for studying the synthesis of glutamylhydroxamic acid consisted of 1 ml. enzyme preparation, 0.044M 4(5)-methylglyoxaline buffer, pH 7.5, 0.01M-MgSO₄, 0.11M sodium L-glutamate, 0.44M hydroxylamine, 0.006M ATP in a total volume of 4.5 ml. For glutamine synthesis, the hydroxylamine was replaced by 14.3 μ moles NH₄Cl. Control systems contained no added glutamate. The pH of solutions of glutamic acid and hydroxylamine were adjusted during their preparation to that of the buffer in the experiment concerned.

The microbiological assay procedure for the identification and estimation of glutamine was that of Roper & Mellwain (1948).

Results are expressed in terms of μ moles/total volume of experimental system.

RESULTS

Preliminary experiments

A washed suspension of *M. pyogenes* var. *aureus* was disintegrated in the Mickle machine and then centrifuged at 1000 g. After removing the supernatant fluid, the insoluble material was suspended in 0.9% (w/v) NaCl (volume equivalent to half that of the original suspension). The original observations of Elliott & Gale (1948) that the supernatant catalysed the synthesis of glutamylhydroxamic acid were confirmed. The suspension of insoluble material showed no activity and synthesis by whole cells was negligible (Table 1). The latter result was not unexpected since Gale (1947) has shown that glutamic acid can only enter *M. pyogenes* when the cells are provided with a utilizable energy source such as glucose. If, however, the normal permeability properties of the cells are destroyed by a detergent such as Aerosol OT (cf. the effect of tyrocidin and phenol; Gale & Taylor, 1947), then synthesis can be observed with whole cells (Table 2). Aerosol in concentrations of less than 2 mg./ml. had no inhibitory effect on the synthesis of glutamylhydroxamic acid

Table 2. *Synthesis of glutamylhydroxamic acid by acetone powder and whole cell suspension pretreated with Aerosol*

In (a) complete system contained 30 mg. acetone powder, 0.44M-NH₂OH, 0.04M potassium phthalate buffer, pH 6.3, 0.04M-MgCl₂, 0.05M sodium glutamate, 0.006M ATP; total vol., 4.5 ml. In (b) 1 ml. washed cell suspension (30 mg. dry wt./ml.) first incubated with 1 ml. 0.2M potassium phthalate buffer, pH 6.3, 0.5 ml. Aerosol (10 mg./ml.), 0.1 ml. 2M-MgCl₂ for 20 min. at 37° and then sodium glutamate, NH₂OH and ATP added as in (a) for complete system. Control systems contained no glutamate. Incubated 1 hr. at 37°.

Enzyme preparation	Hydroxamic acid (μ moles/4.5 ml.)		
	Control (A)	Complete system (B)	Activity (B - A)
(a) Acetone powder	2.3	6.4	4.1
(b) Washed cell suspension			
Untreated	0.6	0.6	0.0
Aerosol treated	0.9	4.1	3.2

by the cell-free system. Since incubation of the enzyme preparation (Mickle) with a number of other compounds did not lead to the formation of a hydroxamic acid, it was concluded that the one formed in the presence of L-glutamic acid was glutamylhydroxamic acid. The substances tested included α -oxoglutarate, oxaloacetate, succinate, fumarate, L-malate, L-glutamine, D-glutamate, L-aspartate, DL-alanine, L-arginine, L-serine, DL-threonine, glycine and DL-phenylalanine at a final concentration of 0.05M. In the range of concentrations studied, the rate of synthesis of glutamylhydroxamic acid was directly proportional to the amount of enzyme preparation (Fig. 1).

The crude enzyme preparation contains phosphatases which rapidly hydrolyse ATP, but attempts to separate such enzymes from the system catalysing the synthesis of glutamylhydroxamic acid met with little success. The methods used in the purification studies comprised isoelectric precipitation with acetate buffers at various pH values and ionic strengths; precipitation in the cold by ethanol or acetone; heating in the presence and absence of cofactors; adsorption on to calcium phosphate gel

and elution by salt solutions; and fractionation with $(\text{NH}_4)_2\text{SO}_4$. The most promising results were obtained by adsorption of the enzyme system on to calcium phosphate gel at pH 6.3 and then eluting it from the gel with 9% (w/v) Na_2SO_4 in 0.02M potassium phthalate buffer, pH 6.3. In the $(\text{NH}_4)_2\text{SO}_4$ fractionation of the eluate, the fraction precipitating at 50–55% saturation contained a large proportion of the required enzyme system and only a small amount of phosphatase activity. Unfortunately the enzyme was now in an unstable state, and within 2 hr. from the time of preparation, half the activity had been lost. Inactivation could not be prevented by the addition of cysteine, glutathione, Mg^{2+} , boiled yeast extract or by altering the pH. The analogous system from sheep brain is also unstable after it has been subjected to various purification procedures, and no method for stabilizing the purified preparations was discovered (Elliott, 1951). However, unlike the bacterial enzyme preparation, the cell-free extract of acetone powders of sheep brain exhibited little phosphatase activity. Acetone powders of *M. pyogenes* also contain the enzyme system in an active state (Table 2), but no way could be found of extracting the enzyme system without the phosphatases.

Since attempts to obtain a purified preparation were unsuccessful, the general properties and kinetics of the enzyme system were studied using the crude cell-free extracts prepared by disintegration of suspensions in the Mickle machine.

Fig. 1. Effect of enzyme concentration on rate of synthesis of glutamylhydroxamic acid. Incubated 1 hr. at pH 6.3 (●) and 20 min. at pH 7.5 (○); temp., 37°. For each enzyme concentration, complete systems contained 0.04M potassium phthalate buffer, pH 6.3, or 0.04M 4(5)-methylglyoxaline buffer, pH 7.5, 0.44M NH_2OH , 0.04M MgCl_2 , 0.05M sodium glutamate, 0.006M ATP; total vol., 4.5 ml. Control systems, no glutamate. Values in controls subtracted.

Synthesis of a compound containing an amide group

McIlwain (1948) has shown that washed cell suspensions of staphylococci decompose glutamine at a slow but significant rate. It was therefore feasible that the enzyme preparation (Mickle) possessed glutaminase activity and this would have complicated any measurement of glutamine synthesis. However, when the enzyme preparation was incubated with glutamine at pH 5.5 (0.04M Na acetate buffer), 6.5 and 7.5 (0.04M K phosphate buffer (Sørensen, 1912)), no hydrolysis of the amide group could be detected, though the washed cell suspensions from which the cell-free extracts were prepared did show glutaminase activity.

The enzyme preparation itself contains free NH_3 and even in the absence of added NH_4Cl , incubation in the presence of ATP, L-glutamate and Mg^{2+} (Table 3) resulted in a decrease (1.6 μmoles) in the free NH_3 of the system. This decrease was correlated with an increase (1.4 μmoles) in the amount of NH_3 which was released in conditions known to hydrolyse the amide group of glutamine, i.e. 5% H_2SO_4 at 100° for 10 min. (Krebs, 1935). Since glutamine and isoglutamine (and possibly asparagine) are the only amides likely to be formed in such experimental conditions, and since isoglutamine and asparagine are much more resistant to acid hydrolysis, there was good reason to believe that the cell-free extracts of *M. pyogenes* catalysed the synthesis of glutamine. The enzyme system evidently has a high affinity for NH_3 (see later), but to ensure that glutamine synthesis was not limited by the amount of NH_3 available, 14.3 μmoles NH_4Cl /4.5 ml. of experimental system were added in all subsequent experiments.

Identification of the amide as glutamine. Glutamine can be assayed in the presence of glutamic acid and ammonia by using the microbiological assay method introduced by Roper & McIlwain (1948). When freshly isolated, *Strep. haemolyticus* (Lancefield group A) requires glutamine for growth, though in some circumstances this requirement can be replaced by much greater amounts of glutamic acid. Utilization of the latter in place of glutamine is

Table 3. *Synthesis of an amide by the cell-free extract*

Complete system: 2 ml. enzyme preparation (Mickle), 0.04M MgCl_2 , 0.11M sodium glutamate (GA), 0.004M ATP, 0.04M potassium phthalate buffer, pH 6.3; total vol., 4.5 ml. Incubated 15 min. at 37°.

	Ammonia (μmoles)			
	Complete system	No ATP	No Mg^{2+}	No GA
Free NH_3	6.3	7.9	—	—
NH_3 released by acid hydrolysis (=amide NH_2)	1.8	0.4	0.0	0.5

prevented by including methionine sulphoxide in the medium. In the complete experimental system, L-glutamate, ATP, Mg^{2+} and NH_3 were incubated with the enzyme preparation at pH 7.5. A second system contained no added glutamic acid and served as a control. After 1 hr. at 37° the reaction was stopped by cooling in an ice bath, and the amount of amide in the two systems was determined by the analysis of 3 ml. samples using the Parnas procedure. Other samples (1 ml.) were brought to pH 7, diluted to a known volume, sterilized by filtration through Seitz filters, and then assayed microbiologically for their glutamine content. After incubation at 37° for 24 hr. turbidity was determined. Growth had occurred only in the tubes to which samples from the complete experimental system had been added, and the amount of glut-

amine producing similar growth was equivalent to the amide content as estimated by the Parnas method (Table 4). Since glutamine is the only compound known to promote the growth of *Strep. haemolyticus* in the medium used (cf. McIlwain, 1939), it was concluded that the results of the microbiological assay, together with the acid hydrolysis data showed that the amide synthesized by the enzyme preparation from *M. pyogenes* was glutamine.

Synthesis of glutamylhydroxamic acid and glutamine

Progress curves and effect of pH. The progress of the synthesis of glutamylhydroxamic acid and glutamine by the same enzyme preparation was determined at pH 6.3, samples of the experimental systems being taken at intervals (Fig. 2). The

Table 4. Microbiological assay of the amide

Complete and control experimental systems: the standard systems (Methods, p. 581). Incubated 1 hr. at 37°. 3 ml. of experimental system taken for determination of amide by Parnas method. In the microbiological assay, 2 and 4 ml. of a 1:10 dilution of 1 ml. of the control system and 2, 3 and 4 ml. of a 1:100 dilution of 1 ml. of the complete system were used.

	Amounts found (μ m-moles/ml.)			
	Amide by Parnas method		Growth response in terms of glutamine	
	Control system	Complete system	Control system	Complete system
Initially (a)	66	66	0	0
Finally (b)	66	966	0	820, 850, 860
Synthesis (b - a)	0	900	0	Mean, 840

Fig. 2. Progress of synthesis of glutamylhydroxamic acid and glutamine. Results expressed as μ moles/4.5 ml. experimental system after subtraction of values in controls. Complete systems: 5 ml. enzyme preparation (Mickle), 0.4M potassium phthalate buffer, pH 6.3, 0.04M-MgCl₂, 0.1M sodium glutamate, 0.006M ATP together with 0.4M-NH₂OH for synthesis of hydroxamic acid (●) or 0.006M-(NH₄)₂SO₄ for synthesis of glutamine (○); total vol., 30 ml. Control systems, no glutamate; temp., 37°; 4.5 ml. samples withdrawn at intervals.

Fig. 3. Effect of pH on rate of synthesis of glutamylhydroxamic acid and glutamine. For synthesis of hydroxamic acid (●), complete systems contained 1 ml. enzyme preparation (Mickle), 0.04M buffer, 0.4M-NH₂OH, 0.04M-MgCl₂, 0.1M sodium glutamate, 0.006M ATP; total vol., 4.5 ml. For synthesis of glutamine (○), NH₂OH replaced by 0.003M-(NH₄)₂SO₄. Control systems, no glutamate. Incubated 1 hr. at 37°. Buffers: 4-hydroxymethylglyoxaline at pH 5.5, 6.0 and 6.5; 4(5)-methylglyoxaline at pH 7.0 and 7.5; 2,4-dimethylglyoxaline at pH 8.0. Values in controls subtracted.

increase in the amount of glutamine was closely paralleled by the increase in glutamylhydroxamic acid. The effect of hydrogen-ion concentration was studied using glyoxaline buffers covering the pH range 5.5–8.0 (Fig. 3). The optimum pH for the synthesis of glutamylhydroxamic acid and glutamine was in the region of 7.5, although the optimum for glutamine was not as sharply defined as that for glutamylhydroxamic acid. Similar observations were made by Speck (1949) with the pigeon-liver system, in which the rate of glutamine synthesis showed little variation in the pH range 7.5–8.5, whereas the optimum for glutamylhydroxamic acid was sharply defined at pH 7.0.

Since glutamylhydroxamic acid was synthesized at the same rate as glutamine, and, except at a pH greater than 7.5, the pH influenced the rate of synthesis of each of these compounds to an equal extent, it was concluded that the same enzyme system is involved in the synthesis of both of these substances. For this reason, and because of a more convenient method of estimation, the effects of some experimental conditions were studied only in terms of glutamylhydroxamic acid, and it was assumed that they would affect glutamine synthesis in a similar manner. All subsequent experiments were performed at the optimum pH of 7.5. At this pH, as at pH 6.3, the rate of synthesis of glutamylhydroxamic acid was directly proportional to the amount of enzyme preparation, i.e. in the range of concentrations examined (Fig. 1).

Fig. 4. Effect of concentration of magnesium or manganous ions on rate of synthesis of glutamylhydroxamic acid. Complete systems: 0.5 ml. enzyme preparation (Mickle), 0.04M 4(5)-methylglyoxaline buffer, pH 7.5, 0.4M-NH₂OH, MgSO₄ (○) or MnSO₄ (●), 0.1M sodium glutamate, 0.006M ATP; total volume, 4.5 ml. Control systems: no glutamate. Incubated 30 min. at 37°. Values in controls subtracted.

Effect of cations. Synthesis of glutamylhydroxamic acid is negligible unless the system is fortified with Mg²⁺ (Table 1). Attempts were made to replace Mg²⁺ by Ca²⁺, Sr²⁺, Ba²⁺, Zn²⁺, UO₂²⁺, Na⁺, K⁺, Mn²⁺, Fe²⁺ or Fe³⁺ at a final concentration of 0.01M and 0.001M. Of these cations, only Mn²⁺ showed any activity. In low concentrations, Mn²⁺ was more effective than Mg²⁺, and the maximum rate of synthesis, achieved in the presence of 0.0014M-Mn²⁺, was the same as that with an optimal concentration of Mg²⁺ (0.01M) (Fig. 4). With concentrations of Mn²⁺ greater than 0.0014M, there was progressive inhibition, though no inhibitory effects were observed with concentrations of Mg²⁺ up to 0.04M. Ca²⁺ antagonized the activating effect of Mg²⁺ and Mn²⁺, and with increasing concentrations of Ca²⁺ there was progressive inhibition of the synthesis of glutamylhydroxamic acid (Fig. 5). The concentration of Ca²⁺ producing 50% inhibition was the same, irrespective of whether the system was activated by Mg²⁺ or Mn²⁺, although the concentration required for complete inhibition was greater when Mn²⁺ was present. The latter effect may be correlated with the greater affinity of the enzyme system for lower concentrations of Mn²⁺ than Mg²⁺. The inhibitory action of Ca²⁺ in the presence of Mg²⁺ was competitive, and increasing the concentration of Mg²⁺ decreased the inhibition due to a particular concentration of Ca²⁺ (Table 7).

Fig. 5. Effect of calcium ions on the synthesis of glutamylhydroxamic acid in the presence of magnesium or manganous ions. Complete systems: 1 ml. enzyme preparation (Mickle), 0.4M-NH₂OH, 0.04M 4(5)-methylglyoxaline buffer, pH 7.5, either 0.04M-MgCl₂ (●) or 0.0016M-MnSO₄ (○), 0.1M sodium glutamate, 0.007M ATP. Ca²⁺ added as CaCl₂. Total vol., 5 ml. Control systems: no glutamate. Incubated 45 min. at 37°. Values in controls subtracted. Results compared with synthesis in absence of Ca²⁺ and expressed as percentage inhibition.

Effect of concentration of reactants. In a series of experiments, the rate of synthesis of glutamylhydroxamic acid was determined in experimental systems in which the concentration of one of the reactants was varied. The graphs relating rate of synthesis with concentration of reactant (ATP, glutamate or hydroxylamine) were of the typical Michaelis-Menten type, and the Michaelis constants (K_m) for ATP and glutamate were determined by the method of Lineweaver & Burk (1934) (Figs. 6, 7). Although the system has a high affinity for hydroxylamine, a relatively large concentration is required to achieve the maximum rate of synthesis (Fig. 8), and it is therefore more convenient to express the results in terms of rate and substrate concentration rather than as reciprocals of these quantities. Under the experimental conditions employed, the Michaelis constants for ATP, glutamate and hydroxylamine were of the order 0.0028, 0.011 and 0.003 M, respectively. The concentrations of ATP, glutamate and hydroxylamine

Fig. 6. Effect of concentration of ATP on rate of synthesis of glutamylhydroxamic acid. Complete systems: 1 ml. enzyme preparation (Mickle), 0.4 M-NH₂OH, 0.04 M 4(5)-methylglyoxaline buffer, pH 7.5, 0.01 M-MgCl₂, 0.1 M sodium glutamate, ATP at molar concentrations S . Control systems: no glutamate. Values in controls subtracted. v = μ mole hydroxamic acid synthesized in 20 min. at 37°.

Fig. 7. Effect of glutamate concentration on rate of synthesis of glutamylhydroxamic acid. Complete systems: 1 ml. enzyme preparation (Mickle), 0.04 M 4(5)-methylglyoxaline buffer, pH 7.5, 0.4 M-NH₂OH, sodium glutamate at molar concentrations S , 0.04 M-MgCl₂, 0.006 M ATP; total vol., 4.5 ml. Control systems: no glutamate. Values in controls subtracted. v = μ mole hydroxamic acid synthesized in 20 min. at 37°.

Fig. 8. Effect of hydroxylamine concentration on the rate of synthesis of glutamylhydroxamic acid. Complete systems: 1 ml. enzyme preparation (Mickle), 0.04 M 4(5)-methylglyoxaline buffer, pH 7.5, NH₂OH, 0.01 M-MgCl₂, 0.1 M sodium glutamate, 0.006 M ATP; total vol., 4.5 ml. Control systems, no glutamate. Values in controls subtracted. Incubated 30 min. at 37°.

Table 5. *Effect of ammonia concentration on glutamine synthesis*

Control systems (1 and 2): no glutamate. Complete systems (3–7) were, except for added NH₄Cl, the standard experimental systems (see Methods, p. 581). The enzyme preparation (Mickle) contained 4.8 μ moles NH₃/ml. Amide determined as ammonia released by acid hydrolysis. Amide initially in system, 0.4 μ mole/4.5 ml. Temp. 37°.

	No glutamate		Complete systems				
	1	2	3	4	5	6	7
Total concn. of NH ₃ at zero time (M $\times 10^3$)	1.1	7.4	1.1	1.9	2.7	4.3	7.4
Amide after 30 min. (μ moles)	0.4	0.4	3.2	3.2	3.3	3.2	3.1
Amide synthesized (μ moles)	0.0	0.0	2.8	2.8	2.9	2.8	2.7

Table 6. *Liberation of phosphate during synthesis of glutamylhydroxamic acid and glutamine*

(a) Complete system: total volume 30 ml. containing 6 ml. enzyme preparation (Mickle), 0.44M-NH₂OH, 0.04M 4(5)-methylglyoxaline buffer, pH 7.5, 0.04M-MgCl₂, 0.1M sodium glutamate and 0.007M ATP. Samples (5 ml.) taken at intervals and reaction stopped by 1 ml. 40% (w/v) trichloroacetic acid; 4.5 ml. taken for hydroxamic acid and 1 ml. for phosphate determinations. Control: no glutamate; temp., 37°.

Time (min.)	Amounts found (μmoles/5 ml.)					
	Hydroxamic acid			Phosphate		
	Control (A)	Complete system (B)	Activity (B - A)	Control (A)	Complete system (B)	Activity (B - A)
0	0.8	0.8	0.0	7.4	7.4	0.0
15	0.8	5.9	5.1	10.3	15.2	4.9
30	0.9	8.9	8.0	12.3	20.7	8.4
45	0.9	10.1	9.2	14.5	24.2	9.7

(b) Complete system: in 5 ml., 1 ml. of enzyme preparation (Mickle), 14.3 μmoles NH₄Cl, with ATP, MgCl₂, sodium glutamate and buffer as in (a). Control: no glutamate. Reaction stopped as in (a); 4 ml. taken for amide determination (as NH₃ released by acid hydrolysis), 1 ml. for phosphate determination. Duration of expt. 45 min.

	Amounts found (μmoles/5 ml.)		
	Control (A)	Complete system (B)	Activity (B - A)
Amide	1.4	10.3	8.9
Phosphate	16.4	26.3	9.9

Fig. 9. Effect of sodium fluoride on the synthesis of glutamylhydroxamic acid in the presence of magnesium or manganous ions. Complete systems: 1 ml. enzyme preparation (Mickle), 0.4M-NH₂OH, 0.04M 4(5)-methylglyoxaline buffer, pH 7.5, either 0.04M-MgCl₂ (○) or 0.0016M-MnSO₄ (●), 0.1M sodium glutamate, 0.007M ATP and NaF; total vol., 5 ml. Control systems: no glutamate. Incubated 1 hr. at 37°. Values in controls subtracted. Results compared with synthesis in the absence of NaF and expressed as percentage inhibition.

required for the maximum rate of synthesis were of the order 0.007, 0.055 and 0.1M, respectively. When the rate of synthesis of glutamine was studied as a function of NH₃ concentration it was found that

with short incubation periods the amount of NH₃ already present in the enzyme preparation was sufficient for the maximum rate of synthesis (Table 5). Hence the affinity of the enzyme system for NH₃ is high, and since in the reported experiment (Table 5) synthesis was maximal in the presence of 4.8 μmoles NH₃/4.5 ml., *K_m* was calculated to be less than 0.001M.

Synthesis of glutamylhydroxamic acid or glutamine and the appearance of phosphate. As the synthesis of glutamylhydroxamic acid proceeds, there is an increase in the amount of inorganic phosphate in the system (Table 6). The amounts of phosphate liberated and of hydroxamic acid synthesized agree to within 6%, indicating that 1 mole of ATP is utilized in the synthesis of 1 mole of the hydroxamic acid. The synthesis of glutamine by the same enzyme preparation is also accompanied by the appearance of an equivalent amount of phosphate (Table 6).

Inhibitors. NaF inhibits the synthesis of glutamylhydroxamic acid and glutamine, the degree of inhibition being influenced by the activating cation in the system. Much higher concentrations were required to inhibit the synthesis of glutamylhydroxamic acid in the presence of Mn²⁺ than in the presence of Mg²⁺ (Fig. 9), e.g. 3.35 × 10⁻⁴M-NaF produced 50% inhibition when Mg²⁺ was the activating ion, but for the same degree of inhibition, a concentration of 1.3 × 10⁻²M was required when the system was activated by Mn²⁺.

p-Chloromercuribenzoate is a potent inhibitor of the synthesis of glutamylhydroxamic acid (Fig. 10), 50 % inhibition being produced by a concentration of 3.8×10^{-5} M. Cysteine protects the system from the effects of the inhibitor, e.g. in one experiment 5.6×10^{-4} M *p*-chloromercuribenzoate brought about 67 % inhibition of glutamylhydroxamic acid synthesis: if cysteine (final concentration 0.01 M) was added before or after the inhibitor, the latter now had no effect, and synthesis was the same as in its absence. Since this inhibitor reacts with thiol groups (Hellerman, Chinard & Deitz, 1943), these observations indicate that such groups play an

important part in the synthesis of glutamylhydroxamic acid, though whether they are located in the protein part of the enzyme system or in a coenzyme (such as coenzyme A) is not known.

Elliott & Gale (1948) showed that methionine sulphoxide was a competitive inhibitor of the synthesis of glutamylhydroxamic acid, and their conclusion that it was a competitive inhibitor of glutamine synthesis (cf. Borek, Miller, Sheiness & Waelsch, 1946) was confirmed (Fig. 11). The inhibitory properties of methionine sulphone were identical with those of the sulphoxide. A number of other compounds were examined, and of these only adenosine diphosphate (ADP) possessed any

Fig. 10. Inhibition of synthesis of glutamylhydroxamic acid by *p*-chloromercuribenzoate. Complete and control systems: the standard experimental systems (Methods, p. 581) with *p*-chloromercuribenzoate added to both as required. Incubated 1 hr. at 37°. Values in controls subtracted. Results compared with synthesis in the absence of inhibitor and expressed as percentage inhibition.

Fig. 11. Inhibition of glutamine synthesis by methionine sulphoxide. Complete and control systems: the standard experimental systems (Methods, p. 581) with glutamate in molar concentrations *S* in the presence (O) and absence (●) of 0.007 M methionine sulphoxide. Values in controls subtracted. *v* = μ moles hydroxamic acid synthesized in 1 hr. at 37°.

Table 7. *Effect of antibiotics, ADP, aspartate and other substances on the synthesis of glutamylhydroxamic acid (Glu. HA) or glutamine (Glu. NH₂)*

Complete and control systems: the standard systems (see Methods, p. 581) with modifications shown in Table. Incubated 1 hr. at 37°.

Substance	Concn.	Activating cation	Synthesis of Glu. HA or Glu. NH ₂	Inhibition (%)
Gramicidin	1 mg./ml.	Mg ²⁺	Glu. HA	0
Penicillin	100 μ g./ml.	Mg ²⁺	Glu. HA	0
NaN ₃	0.01 M	Mg ²⁺	Glu. HA	0
2:4-Dinitrophenol	0.002 M	Mg ²⁺	Glu. HA	0
NaN ₃	0.01 M	Mn ²⁺	Glu. HA	0*
2:4-Dinitrophenol	0.002 M	Mn ²⁺	Glu. HA	0
NaCN	0.01 M	Mn ²⁺	Glu. HA	0†
NaF	0.00034 M	Mg ²⁺	Glu. NH ₂	47
NaF	0.00034 M	Mn ²⁺	Glu. NH ₂	21
CaCl ₂	0.001 M	(0.0014 M) Mg ²⁺	Glu. HA	61
CaCl ₂	0.001 M	(0.01 M) Mg ²⁺	Glu. HA	0
Na aspartate	0.11 M	(0.05 M) Mg ²⁺	Glu. HA	22
ADP (+ 0.007 M ATP)	0.002 M	Mg ²⁺	Glu. HA	41
ADP (+ 0.014 M ATP)	0.002 M	Mg ²⁺	Glu. HA	31

* Stimulated synthesis by 12%.

† Stimulated synthesis by 14%.

Table 8. *Synthesis of glutamylhydroxamic acid by cell-free extracts of various species of bacteria*

Cell-free extracts prepared by disintegration of washed cell suspensions in the Mickel machine for 45 min. Cultures grown for 6 hr. (except lactobacilli, 16 hr.) at 37°. Figures in parentheses are NCTC numbers. Complete system: standard experimental system (see Methods, p. 581). Incubation period 1 hr. at 37°. $q_{\text{GHA}} = \mu\text{moles hydroxamic acid (A - B)/mg. total N of extract/hr.}$

Organism	Dry wt. of suspension	Glutamylhydroxamic acid formed ($\mu\text{moles/4.5 ml.}$)				q_{GHA}
		Complete system (A)	No glutamate (B)	No ATP	Activity (A - B)	
<i>Bact. cadaveris</i>	40	2.7	0.9	0.3	1.8	0.7
<i>Cl. welchii</i> SR 12	60	2.6	0.6	0.2	2.0	0.8
<i>Esch. coli</i> (86)	30	3.9	2.6	0.6	1.3	0.8
<i>Esch. coli</i> K 4	30	2.9	1.7	9.2	1.2	0.7
<i>Lb. arabinosus</i> 17-5	45	2.5	1.6	0.1	0.9	0.3
<i>Lb. casei</i>	45	2.3	1.8	0.2	0.5	0.2
<i>Ln. mesenteroides</i>	30	1.9	0.7	0.1	1.2	0.7
<i>M. pyogenes</i> var. <i>aureus</i> (563)	30	4.2	1.4	0.1	2.8	2.1
<i>M. pyogenes</i> var. <i>aureus</i> (1560)	30	3.9	0.8	0.2	3.1	2.6
<i>M. pyogenes</i> var. <i>aureus</i> (6773)	30	3.8	1.1	0.4	2.7	2.5
<i>Pr. vulgaris</i>	30	2.6	1.8	0.3	0.8	0.1
<i>Strep. faecalis</i> (6782)	30	2.1	0.8	0.3	1.3	0.9
<i>Strep. faecalis</i> (6783)	30	3.5	1.2	0.2	2.3	1.6

marked inhibitory action (Table 7). The latter was decreased by increasing the concentration of ATP, indicating that ADP competes with ATP for the appropriate reactive centres in the enzyme system. Gramicidin, penicillin and 2:4-dinitrophenol, in the concentrations examined, had no effect. Azide and cyanide tended to increase the rate of synthesis. A similar activating effect by cyanide was observed by Speck (1949) with the pigeon-liver system. Aspartate even when present in a concentration equivalent to the glutamate, only inhibited synthesis to a small extent (Table 7).

Synthesis of glutamylhydroxamic acid by other bacteria. Cell-free extracts of a number of bacteria were prepared by the same method as for *M. pyogenes* var. *aureus* strain Duncan, and then each was incubated in the standard experimental systems to determine whether the preparation catalysed the synthesis of glutamylhydroxamic acid. Extracts from all the organisms examined were able to accomplish this reaction (Table 8). Whilst the activities of the extracts from some species were relatively low, the amounts of hydroxamic acid formed are regarded as being significant. As an aid to comparing the activities of different species, the total nitrogen content/ml. of each enzyme preparation was determined and the results expressed in terms of $\mu\text{moles hydroxamic acid synthesized/hr./mg. total nitrogen}$. For the reasons given above, it was assumed that the demonstration that these organisms were able to synthesize glutamylhydroxamic acid implied that they were also able to synthesize glutamine, though this was only studied and proved directly in the case of *Strep. faecalis*.

DISCUSSION

From the results reported in Tables 3 and 6, it is concluded that the stoichiometry of the synthesis

of glutamine by *M. pyogenes* var. *aureus* is represented by the equation:

With regard to activation by magnesium and manganous ions, the Michaelis constants for the various reactants, the optimum pH, and inhibition by calcium ions, sodium fluoride, ADP and *p*-chloromercuribenzoate, the enzymic system in *M. pyogenes* responsible for the synthesis of glutamine and glutamylhydroxamic acid has many properties in common with analogous systems obtained from sheep brain (Elliott, 1948, 1951), pigeon liver (Speck, 1949) and pea seedlings (Elliott, 1953). A major difference is that crystal violet and other triphenylmethane dyes inhibit glutamine synthesis by *M. pyogenes* var. *aureus* (cf. Elliott & Gale, 1948; Fry, 1949) but not by sheep brain (Elliott, 1948) or pigeon liver (Speck, 1949).

No direct evidence is yet available concerning the detailed mechanism of glutamine synthesis. By analogy with the synthesis of acetylhydroxamic acid and acetylsulphanilamide, it has been suggested (Elliott, 1951; Speck, 1949) that intermediate stages in the reaction may involve either the formation of an energy-rich glutamyl phosphate or a co-factor such as coenzyme A bearing an 'activated' glutamyl radical. Elliott (1951), Speck (1949) and the present author have been unable to obtain any evidence for the production of a glutamyl phosphate, and Speck (1949) has also reported indirect evidence that coenzyme A is not a participant in this reaction. As a result of experiments with ^{32}P , Webster & Varner (1954) have recently proposed that the synthesis involves phosphorylation of the enzyme followed by exchange of the phosphate group for a glutamyl radical, which can then be

transferred to a suitable amino acceptor such as ammonia.

The ability to synthesize glutamine appears to be widespread in bacteria (Table 9), and it is pertinent to note that there are only a few species for which this substance is a growth factor. Moreover, except when small inocula are used, this requirement is not absolute and glutamine can be replaced by glutamic acid (Fildes & Gladstone, 1939; Pollack & Lindner, 1942). Glutamine has been associated with protein synthesis (Fruton, Johnston & Fried, 1951), transfer reactions with ammonia and hydroxylamine (cf. Waelsch, 1952), citrulline synthesis (Ory, Hood & Lyman, 1954) and with maintaining the rate of glucose fermentation in streptococci (McIlwain, Roper & Hughes, 1948). However, as yet, there is no direct information concerning the role of this substance in the intermediary metabolism of micro-organisms. Since γ -glutamyl groups are also found in the folic acid factors (Mowat *et al.* 1948; Boothe *et al.* 1948), glutathione (Harington & Mead, 1935) and perhaps streptogenin (Woolley, 1948), it is possible that part or all of the enzyme system responsible for the synthesis of glutamine may also play a part in the synthesis of these compounds, though Bloch & Johnston (1949) on the basis of their experiments believe that at least in the case of glutathione no such relationship exists.

SUMMARY

1. Cell-free extracts of *M. pyogenes* var. *aureus* and a number of other bacteria catalyse the synthesis of glutamylhydroxamic acid from glutamic acid and hydroxylamine in the presence of ATP and magnesium ions.

2. An amide, identified as glutamine, is synthesized when hydroxylamine is replaced by ammonia.

3. Synthesis of glutamine and glutamylhydroxamic acid is accompanied by the appearance of an equivalent amount of phosphate.

4. The enzyme system is activated by optimum concentrations of manganous ions as effectively as by magnesium ions.

5. Synthesis is inhibited by calcium ions, sodium fluoride, adenosine diphosphate, *p*-chloromercuribenzoate and methionine sulfoxide. Sodium fluoride is more effective in the presence of magnesium ions than in the presence of manganous ions.

The author wishes to thank Dr E. F. Gale, F.R.S., and Dr W. H. Elliott for their continued interest in this work and much valuable advice, and he gratefully acknowledges the receipt of a training grant from the Medical Research Council.

REFERENCES

- Bailey, K. (1942). *Biochem. J.* **36**, 121.
 Bailey, K. (1949). *Biochem. J.* **45**, 479.
 Bloch, K. & Johnston, R. B. (1949). *J. biol. Chem.* **179**, 493.
 Boothe, J. H., Mowat, J. H., Hutchings, B. L., Angier, R. B., Waller, C. W., Stockstad, E. L. R., Semb, J., Gazzola, A. L. & Subbarow, Y. (1948). *J. Amer. chem. Soc.* **70**, 1099.
 Borek, E., Miller, H. K., Sheiness, P. & Waelsch, H. (1946). *J. biol. Chem.* **163**, 347.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.
 Elliott, W. H. (1948). *Nature, Lond.*, **161**, 128.
 Elliott, W. H. (1951). *Biochem. J.* **49**, 106.
 Elliott, W. H. (1953). *J. biol. Chem.* **201**, 661.
 Elliott, W. H. & Gale, E. F. (1948). *Nature, Lond.*, **161**, 129.
 Fildes, P. & Gladstone, G. P. (1939). *Brit. J. exp. Path.* **20**, 334.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Fruton, J. S., Johnston, R. B. & Fried, M. (1951). *J. biol. Chem.* **190**, 39.
 Fry, B. A. (1949). *1st Int. Congr. Biochem. Abstr.* p. 464.
 Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
 Gale, E. F. & Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 77.
 Harington, C. R. & Mead, T. H. (1935). *Biochem. J.* **29**, 1602.
 Hellerman, L., Chinard, F. P. & Deitz, V. R. (1943). *J. biol. Chem.* **147**, 443.
 Hughes, D. E. (1950). *Biochem. J.* **46**, 231.
 Kirby, A. H. M. & Neuberger, A. (1938). *Biochem. J.* **32**, 1146.
 Krebs, H. A. (1935). *Biochem. J.* **29**, 1951.
 LePage, G. A. (1949). In *Manometric techniques and related methods for the study of tissue metabolism*, edited by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. 2nd ed. Minneapolis: Burgess Publishing Co.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21; **161**, 415.
 McIlwain, H. (1939). *Biochem. J.* **33**, 1942.
 McIlwain, H. (1948). *J. gen. Microbiol.* **2**, 186.
 McIlwain, H., Roper, J. A. & Hughes, D. E. (1948). *Biochem. J.* **42**, 492.
 Mickle, H. (1948). *J. Roy. micr. Soc.* **68**, 10.
 Mowat, J. H., Hutchings, B. L., Angier, R. B., Stockstad, E. L. R., Boothe, J. H., Waller, C. W., Semb, J. & Subbarow, Y. (1948). *J. Amer. chem. Soc.* **70**, 1096.
 Ory, R. L., Hood, D. W. & Lyman, C. M. (1954). *J. biol. Chem.* **207**, 267.
 Parnas, J. K. & Heller, J. (1924). *Biochem. Z.* **152**, 1.
 Pirie, N. W. (1930). *Biochem. J.* **24**, 51.
 Pollack, M. A. & Lindner, M. (1942). *J. biol. Chem.* **143**, 655.
 Roper, J. A. & McIlwain, H. (1948). *Biochem. J.* **42**, 485.
 Sørensen, S. P. L. (1912). *Ergebn. Physiol.* **12**, 393.
 Speck, J. F. (1949). *J. biol. Chem.* **179**, 1387, 1405.
 Vickery, H. B., Pucher, G. W. & Clark, H. E. (1935). *J. biol. Chem.* **109**, 39.
 Waelsch, H. (1952). *Advanc. Enzymol.* **13**, 237.
 Webster, G. C. & Varner, J. E. (1954). *J. Amer. chem. Soc.* **76**, 633.
 Woolley, D. W. (1948). *J. biol. Chem.* **172**, 71.