washed with water. This gum gave an H-test showing yellow → greenish-violet (dichroic) colours. It was dissolved in acetic acid (10 ml.) and the cold solution treated with 20% CrO_3 (1 ml.). After 15 min. it was diluted: the partly crystalline precipitate was collected after 2–3 hr., washed, and recrystallized from dilute ethanol. Yield, 0·55 g. of needles, m.p. 185–188°, raised to 190–191° by recrystallization from l.p./benzene and ethyl acetate. (Found: C, 69·3; H, 8·8. Calc. for $C_{29}H_{44}O_7$: C, 69·0; H, 8·8%.) H-test, negative.

This ester (0·3 g.) was heated in a sealed metal bomb for 4 hr. at 200–210° with a solution of Na (0·1 g.) in ethanol (2 ml.) with hydrazine hydrate (0·3 ml. of 95–99%). Acidification of the diluted contents of the cooled bomb precipitated a solid which separated from dilute ethanol as long white needles (0·15 g.) of 7:12-dihydroxy-cholanic acid (III), m.p. 205°.

SUMMARY

1. The series of six possible acids obtainable by the oxidation to carbonyl of one or two secondary hydroxyl groups in natural cholic acid has been completed by the preparation of 12-hydroxy-3:7-diketo-cholanic acid.

2. New derivatives of this acid, of 3:12-dihydroxy-7-keto-, 7:12-dihydroxy-3-keto-, and 3-hydroxy-7:12-diketo-cholanic acids are described; methods for obtaining such substances have been further explored.

The author thanks Allen and Hanburys Ltd. for a gift of cholic acid.

REFERENCES

Gallagher, T. F. & Long, P. (1943). *J. biol. Chem.* **147**, 131.

Haslewood, G. A. D. (1943). *Biochem. J.* **37**, 109.

Lardon, A. & Reichstein, T. (1943). *Helv. chim. Acta*, **26**, 607.

Schmidt, L. H., Hughes, H. B., Green, M. H. & Cooper, E. (1942). *J. biol. Chem.* **145**, 229.

Sihn, T. S. (1938). *J. Biochem. Tokyo*, **27**, 425.

Sobotka, H. (1938). *The Chemistry of the Sterids*, pp. 397, 413, 414. London: Baillière, Tindall and Cox.

A Growth Factor for *C. diphtheriae* Present in Liver

By F. W. CHATTAWAY, FRANK C. HAPPOLD and MARY SANDFORD, *Biochemical Laboratories, School of Medicine, Leeds* 2

(*Received* 11 *December* 1943)

In studies on the nutritional requirements of *C. diphtheriae*, Evans, Handley & Happold (1939) found that a basal medium of known composition including nicotinic acid, pimelic acid and β-alanine (Mueller, 1937 *a*, *b*; Mueller & Cohen, 1937) sufficed for the growth of certain exacting *gravis* strains only with the further addition of certain liver fractions soluble in amyl alcohol, which were themselves replaceable by pantothenic acid. It was shown that these more exacting *gravis* strains, in contradistinction to other strains, were unable to effect the synthesis of pantothenic acid from β-alanine and thus required the complete molecule in their nutrient medium. Subsequently it became apparent that there existed strains, of *intermedius* and *gravis* types, which failed to grow when inoculated lightly into the pantothenate medium but which did so readily upon the further addition of liver concentrates. Representatives of both *intermedius* and *gravis* types have now been used as test organisms and it has become apparent that the new factor or factors required are identical for both the exacting *intermedius* and *gravis* strains. With a *gravis* strain of *C. diphtheriae*, sub-type Dundee, growth occurs in the form of a pellicle, the medium below remaining clear. The pellicle form lends itself to rapid visual estimation suitable for the routine measurement of the growth response to various addenda. We have utilized the growth response of the Dundee organism as the criterion of the presence of active material in liver preparations, and have attempted to concentrate the active substance required by this class of exacting strain (preliminary report: Chattaway, Happold & Sandford, 1943).

EXPERIMENTAL

Inocula. The standard *gravis* strain used throughout was isolated by Prof. Tulloch of Dundee. All stock cultures were maintained on Loeffler medium in the ice-chest. Prior to inoculation of test medium the strains were grown overnight on chocolate agar, after which a small amount of the culture was introduced into approximately 5 ml. of sterile distilled water so that a turbidity was just discernible under the meniscus. After thorough distribution by shaking, inoculations were made with a loopful of the weak suspension.

Basal medium: l-tryptophan, 0·2 g.; *dl*-cystine, 0·2 g.; *dl*-phenylalanine, 0·1 g.; glycine (synthetic), 0·5 g.; *dl*-methionine, 0·1 g.; *dl*-valine, 0·2 g.; *dl*-glutamic acid HCl,

0·5 g.; *dl*-alanine, 0·1 g.; *dl*-leucine, 0·2 g.; *dl*-isoleucine, 0·2 g.; aspartic acid, 0·5 g.; *d*-lysine di-HCl, 0·1 g.; *l*-histidine HCl, 0·1 g.; sodium lactate, 0·9 g.; NaCl, 4·5 g.; K_2HPO_4, 0·84 g.; $MgSO_4.7H_2O$, 0·35 g.; $CuSO_4.5H_2O$, 0·0005 g.; $ZnSO_4.7H_2O$, 0·0004 g.; $MnCl_2.4H_2O$, 0·0002 g.; $FeSO_4.7H_2O$, 0·0001 g.; nicotinic acid, 1000 μg.; β-alanine, 1000 μg.; pimelic acid, 80 μg.; Ca pantothenate, 100 μg. The medium was adjusted to pH 7 and made up to one litre. It was distributed in 5 ml. amounts in $6 \times \frac{3}{4}$ in. test-tubes and autoclaved at 10 lb./sq. in. for 10 min. After cooling, 0·00025 g. $CaCl_2$ was added per tube from a sterile solution.

Other addenda were added in a sterile condition by aseptic technique. After thorough mixing of their contents, the tubes of medium were incubated at 37° for 3 or 4 days. Records of growth were usually obtainable at 30 hr. and onwards and were made as follows:

tr. = A trace of pellicle, just visible to the naked eye and covering less than half the meniscus.

⊥ = A pellicle occupying at least half the surface.

\+ = A pellicle covering thinly the whole of the surface.

++, +++ etc. = Pellicles of varying thickness occupying the entire surface of the liquid.

Active material. Active material was furnished by crude liver concentrates, the earliest of which were prepared by Prof. A. R. Todd and Dr B. Lythgoe of Manchester, in conjunction with one of us (F. C. H.), and constituted a series of which BB31 was the standard fraction. This was a portion of the amyl alcohol insoluble fraction of whole liver (Glaxo Laboratories Ltd., first residues), a solution of which contained 0·276 g./ml. after being dried at 100° and which was equivalent to 23·4 g. of fresh liver/ml. Subsequently preparations of liver, available as by-products in the concentration of the pernicious anaemia factor, were subjected to fractionation procedures in Leeds, constituting the series as set out in Fig. 1. It is possible that these starting materials, ethanol extracts of whole liver, do not constitute the most potent sources.

RESULTS

Stability of active component

(1) *Action of strong acid and alkali.* The stability of the factor varies widely with the hydrogen-ion concentration, being very much greater at pH values below 7 than at those above. Heating liver fractions in either a crude or a relatively purified state, with concentrations of hydrochloric or sulphuric acid up to 20 % for 1½ hr. at 100°, caused no loss in activity. A purified preparation lost 33 % of its potency by heating at 100° for 1 hr. at pH 9, and 90 % after 1½ hr. at pH 11.

(2) *Action of nitrous acid and ninhydrin.* Nitrous acid treatment was found to destroy the active substance. The approximate amount of nitrous acid required in order to ensure an excess was calculated from a determination of the amino-nitrogen by formol titration (amino N/ml. of BB31 = 3 mg.; $NaNO_2$ used = 20 mg./ml.). After standing overnight the treated solution was evaporated to dryness and redissolved in sterile water prior to testing. 70–90 % of the active material of fraction BB31 was destroyed.

The same liver fraction, when boiled for 15 min. with 120 mg. of ninhydrin (triketohydrindene hydrate) in 12 ml. of one-third saturated potassium dihydrogen phosphate solution, developed a deep purple colour and evolved carbon dioxide. High concentrations of the treated liver fraction were inhibitory to growth, although the latter occurred at lower concentrations, but the zone of growth did not extend to the minimum concentrations effective in the case of the untreated liver fractions, corresponding to a loss of about 75 % of the active material. The same was found with the most purified concentrates.

(3) *Methylation and acetylation.* Methylation and acetylation invariably destroyed all activity. Methylation was carried out by refluxing the dried liver preparation for 1 hr. with anhydrous methanol containing 4 % of hydrogen chloride. The acid alone occasioned no loss in potency. All attempts to recover the active material by acid hydrolysis and by mildly alkaline hydrolysis failed.

Acetylation by refluxing the dried liver material with acetic anhydride and pyridine led to the production of inactive, insoluble gummy material. A better method was to treat with successive amounts of ice-cold acetic anhydride and caustic soda. The acetylated product was completely inactive, and potency could not be restored by neutral or acid hydrolysis.

(4) *Oxidation.* The dried liver fraction was taken up in hydrogen peroxide solution ('20 vol.'). After the solution had stood overnight excess hydrogen peroxide was removed by evaporation. This treatment destroyed 70–80 % of the active material.

(5) *Bromine.* 1 ml. of a purified fraction X (Fig. 1) sample was heated with excess bromine (3–4 drops). After standing overnight the halogen was removed by aeration; growth tests revealed no serious fall in potency.

Purification of liver concentrates

Adsorption. It was observed that the active material was readily adsorbed on norite charcoal. Adsorption took place more completely at acid or alkaline reaction, a neutral filtrate retaining considerable activity. Approximately 1·5 g. of norite added to 1 ml. BB31 in 9 ml. water at pH 3 removed practically all the active factor; the adsorbed active material could be quantitatively recovered by elution. At pH 9 the factor was similarly distributed but at pH 6·5 approximately 50 % remained unadsorbed. It was possible to effect some degree of elution by using water at pH 7 but for complete recovery more vigorous methods had to be adopted. A mixture of 50 % ethanol and 10 % NH_3 at 60° was satisfactory, when used repeatedly until no more colour was released from the adsorbent. Practically no activity could be demonstrated in eluates from

fuller's earth adsorptions at pH 3. These observations were used in designing the methods for large-scale concentration.

Extraction with organic solvents. No active material could be extracted by means of petroleum ether, chloroform, or diethyl ether. Amyl alcohol extracted, at pH 1·2, a fair amount of pigment, leaving the active material in the aqueous residue, thus differentiating it sharply from pantothenic acid, which is extracted under these conditions. Preliminary experiments showed that similar results were to be expected with butanol. The effect of the pH of the aqueous phase was tested in more detail. In each case a known volume of BB31 at the required pH was repeatedly extracted with *n*-butanol, the extracts taken to dryness *in vacuo* and redissolved in water to the original volume. At pH values of 1·2, 5·6, 7 and 9 no appreciable amount of the factor entered the organic phase.

Repeated extraction of partially purified liver preparations with *p*-cresol at pH 3 removed about one-third of the solids including the bulk of the pigments, and left the greater part of the activity in the aqueous residue. This is in contrast to the component of our liver concentrates which functions in the stimulation of lactobacilli, and which is extracted by this organic solvent (Chattaway, Happold, Lythgoe, Sandford & Todd, 1943).

Ethanol precipitation. Although more and more activity was precipitated as the ethanol concentration was increased from 70 to 90%, the actual activity/g. of solids remained the same. By employing ethanol concentrations of 50–60%, gross amounts of inorganic impurities were eliminated. In the case of the most purified preparations (fraction X, Fig. 1), we attempted to separate the active component by repeatedly extracting the dried liver material with boiling ethanol. This removed less than half the remaining solids, much organic as well as inorganic matter remaining insoluble. Although at least two-thirds of the active factor was extractable by this procedure, there was a marked diminution of total activity, possibly attributable to that mechanism which caused loss during methylation.

Acetone and dioxane. Acetone in 60–90% (v/v) concentration gave two immiscible layers in the case of fraction X (Fig. 1). At the higher concentration the upper layer was almost colourless and devoid of activity—very little material was extracted. At 60% concentration, activity was equally distributed between the two layers and it seemed likely that repeated extractions would yield useful results. Repeated treatment of a dry purified preparation with hot dioxane showed that the factor is almost insoluble in this solvent.

Barium. Throughout the work baryta was used to eliminate excess sulphate, and vice versa; in no case was there evidence of removal of activity during such manipulations. Ethanol fractionation of barium-treated liver preparations according to the method of Hind, Lythgoe, Macrae, El Sadr, Todd & Work (1939) did not permit purification since the bulk of the material and of the activity remained in the 77% ethanol insoluble fraction.

Silver. Activity resided wholly in the filtrate when liver concentrates were subjected to precipitation with silver nitrate at pH values of 2, 4·5, 7. At pH 9 a small amount of activity was found in the precipitate also. Excess silver was removed either with hydrochloric acid or hydrogen sulphide. Precipitation with silver sulphate by the method of Kossel (1898) was also investigated. The liver fraction was adjusted to pH 4 and treated with excess of a suspension of silver sulphate in water. The precipitate was removed and decomposed with hydrogen sulphide. The filtrate was adjusted to pH 7 with baryta, an excess of silver being maintained. This precipitate was also separated off, acidified with dilute sulphuric acid, filtered and cleared of residual silver with hydrogen sulphide. Similarly a precipitated fraction at pH 9 was obtained. The final filtrate from these three precipitates was acidified with sulphuric acid, filtered and treated with hydrogen sulphide. The original activity of the fraction was found to be present in the final filtrate and to a small extent in the precipitate formed at pH 9. Precipitation could not be made more complete by carrying through the process at more alkaline reaction. Thus it would appear that the active component does not form an insoluble silver derivative but is to some extent adsorbed on to inert material at pH values above 8.

Mercury. The filtrate fractions were fully active when fraction VII (Fig. 1) at pH 6·8 was precipitated with mercuric sulphate or mercurous chloride, the mercury being removed with hydrogen sulphide.

Lead. Liver concentrates were precipitated with excess lead acetate solution at pH values of 2·8, 6·9 and, in the presence of baryta, at pH 9–10. In all cases the activity was predominantly in the filtrate fractions. When hydrogen sulphide was used to remove lead some active material could be recovered from the lead sulphide by elution with ammonia. In other experiments the use of basic lead acetate and ammonia was found partially to precipitate the factor. The precipitation has, however, never been complete and was probably due, as in the case of silver at alkaline reactions, to adsorption rather than salt formation.

Other precipitants. The active substance was not precipitated by: platinic chloride; brucine in 35% ethanol at 0° for a week; saturation with ammonium sulphate; reinecke acid (Dakin & West, 1935); rhodanilic acid; picric acid; flavianic acid; dimedone.

Phosphotungstic acid gave variable results with crude preparations; with purified preparations, however, the factor remained consistently in the filtrate, without serious reduction in potency. Phosphotungstic acid used as a 50% solution in 5% sulphuric acid removed considerable quantities of inert material, including much pigment. Excess reagent was eliminated by making the solution alkaline with baryta, filtering, and acidifying with sulphuric acid as rapidly as possible in order simultaneously to correct the unfavourable reaction and to remove barium.

Since it was found that fraction XI contained sugar and perhaps other reducing material, the effects of 2:4 dinitrophenylhydrazine and of phenylhydrazine were investigated. Removal of the insoluble derivatives obtained did not result in any loss of activity.

Concentration of the growth factor from liver

A method of concentration suitable for large-scale work was designed on the basis of the information obtained in the preliminary examination already recorded. The steps in the method are recorded in Fig. 1. The ethanol concentrates (fraction I) from 1100 kg. fresh liver were diluted with water and extracted with light petroleum (b.p. 40–60°) until no more material was removed. The residue (fraction II) was diluted to 20 l., brought to pH 3 with sulphuric acid, shaken for 1 hr. with 4 kg. of fuller's earth, filtered, the adsorbent washed with acidified

water and the combined washings and filtrate (fraction III) shaken at pH 3 for 1 hr. with successive amounts (2·7 kg. each time) of norite charcoal until no active material remained in the filtrate. The charcoal + adsorbate (fraction IV) was suspended in a mixture of 50 % (v/v) ethanol and 10 % NH_3, whole fraction was saturated with ammonium sulphate and filtered; from the filtrate SO_4^{--} was removed with baryta, and NH_3 by distillation. The concentrate (fraction VI) at pH 3 was extracted three times with 900 g. *p*-cresol; the aqueous residue (fraction VII) at pH 4 was heated with a hot

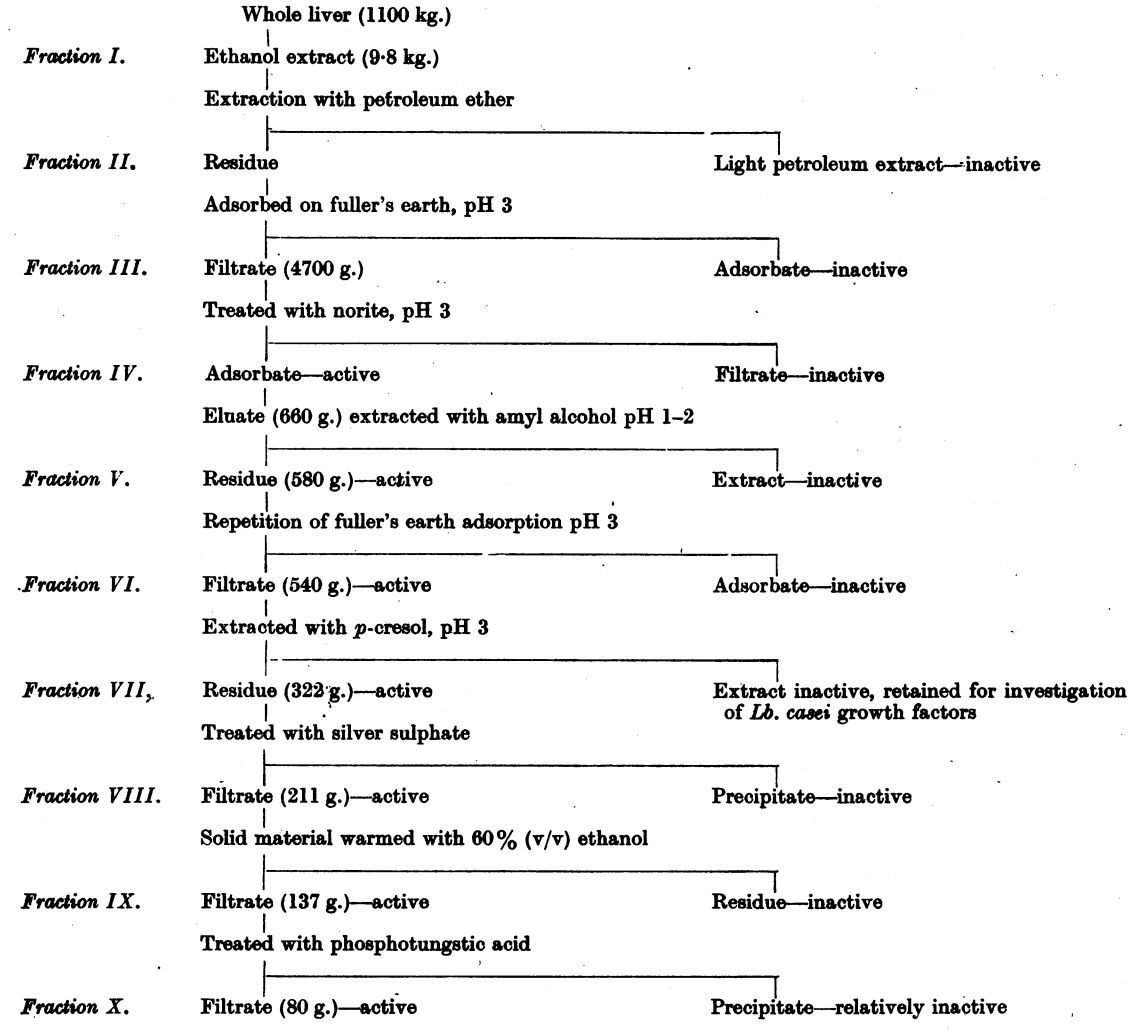

Fig. 1. Concentration of the growth factor from liver.

warmed to 60°, stirred for 30 min., filtered at once and the filtrate distilled *in vacuo*. This elution was repeated until no further active material was removed. The combined eluates were concentrated *in vacuo*, adjusted to pH 1·2 with sulphuric acid, repeatedly extracted with amyl alcohol, the aqueous residue (fraction V) freed from reagent, adjusted to pH 3 with baryta and the fuller's earth adsorption repeated, using 360 g. fuller's earth. A small amount of the concentrated filtrate from the second fuller's earth adsorption was then saturated with ammonium sulphate; if a precipitate formed, the aqueous suspension of sliver sulphate, filtered, the filtrate adjusted to pH 7, silver being maintained in excess, silver removed from the filtrate by hydrogen sulphide and the filtrate (fraction VIII) concentrated to dryness *in vacuo*. The residue was warmed with 60 % (v/v) ethanol (250 ml.), left to stand in ice-chest, filtered. Ethanol was removed from filtrate (fraction IX) which was then treated with a mixture of 50 % phosphotungstic acid and 5 % sulphuric acid in excess. The mixture was then filtered and the filtrate treated with excess baryta and the necessary amount of sulphuric acid, yielding

fraction X. A portion of the latter (36 g.) was extracted with absolute ethanol until no more material was removed (fraction XI; 16 g.). Fraction XI contained total N (micro-Kjeldahl) equivalent to 6% w/w, formol-N 0·5%, total phosphorus (Fiske-Subbarow) 0·16%, no sulphur. This fraction contained carbohydrate not exclusively in free state, since the amount extracted could be increased by acid hydrolysis. Fraction X promoted good growth in concentration of 5 μg./ml. basal medium; 0·05 μg./ml. showed detectable growth in 72 hr.

DISCUSSION

Although the nutritional requirements of many strains of *C. diphtheriae* have been extensively studied, the identification of a further growth factor must be made before certain more exacting strains can be cultivated from light inoculation into a medium of known composition. Whilst some strains of *gravis* and *intermedius* types are entirely dependent upon the supply of this factor, evidence has been obtained that its importance is not limited to these organisms but that it is capable of exciting a marked stimulation with strains of all three types.

Several compounds have been tested in attempts to simulate the effect of the liver concentrates, including: biotin, *p*-aminobenzoic acid, inositol, choline, adenosine, adenylic acid, adenine, guanine, uracil, cytosine, xanthopterin and amino-acids not included in the basal medium. The distinction between the present factor and the stimulant for *Lb. casei*, which accompany each other during the first stages in purification, has already been mentioned. The *Lb. casei* factor resembles in its other properties folic acid as described by Hutchings, Bohonos & Peterson (1941), and it follows that the present *C. diphtheriae* factor cannot be identified with folic acid. A sample of the latter provided by Prof. R. J. Williams had no influence upon growth of our Dundee test organism, either alone or with the addition of active preparations.

It seems probable that the new growth factor cannot be identified with any substance of recognized physiological importance. Its properties indicate certain structural features; its relative simplicity is exemplified by its dialyzability, its resistance to enzymes such as pepsin, trypsin and taka-diastase and its solubility in saturated salt solutions. The molecule is predominantly neither basic nor acidic; the adsorption properties suggest that the isoelectric point lies in the region of neutrality. The factor undergoes loss of physiological function upon methylation and acetylation, the loss being due either to the inactivity of the methyl or acetyl derivatives or to general instability of the factor towards these reagents. The procedure employed during methylation is such that in all probability substitution is possible only at a carboxyl group; the fact that reactivation does not occur on hydrolysis indicates that the acid alcohol exerts other action upon the molecule. The resistance to hydrolysis of the acetylated product is perhaps suggestive of *N*-acetyl groups. Other evidence for the presence of nitrogenous groups is furnished by the results with nitrous acid and ninhydrin, the latter reacting with substances having the molecular construction

$$R\text{—}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{—}COOH \quad \text{and} \quad R\text{—}\underset{\displaystyle NHCH_2R'}{\underset{|}{CH}}\text{—}COOH$$

(Dillon, Hamilton, MacFadyen & Van Slyke, 1941). Although certain of the liver constituents react with ninhydrin in the manner indicated by Van Slyke, there remains the possibility that inactivation may be due to some other mechanism such as oxidation (Rinehart & West, 1942).

SUMMARY

1. Evidence is presented that certain *gravis* and *intermedius* strains of *C. diphtheriae* require a further unidentified growth factor when cultivated in a medium of known composition.

2. The new growth factor is present in whole liver; processes used in its concentration from this source are described.

3. The properties of the factor and their relation to possible structural groupings are discussed.

We acknowledge our indebtedness to Profs. W. J. Tulloch and J. W. McLeod for strains of *C. diphtheriae*, to Prof. A. R. Todd and Dr B. Lythgoe for certain liver fractions (Glaxo Laboratories Ltd.), to Prof. R. J. Williams for a sample of folic acid, and to Mrs D. E. Dolby for polarimetric determinations. Sincere thanks must be expressed to Messrs Rowntree and Co., Ltd., York for a research scholarship to one of us (M. S.) and to the Medical Research Council for a grant (made to F. C. H.). Boots Pure Drug Co. Ltd. kindly supplied the crude liver extracts.

REFERENCES

Chattaway, F. W., Happold, F. C., Lythgoe, B., Sandford, M. & Todd, A. R. (1943). *Nature, Lond.*, **151**, 559.
—— —— & Sandford, M. (1943). *Proc. Biochem. Soc.* **37**, xvi.
Dakin, H. D. & West, R. (1935). *J. biol. Chem.* **109**, 504.
Dillon, R. T., Hamilton, P., MacFadyen, D. A. & Van Slyke, D. D. (1941). *J. biol. Chem.* **141**, 627.
Evans, W. C., Handley, W. R. C. & Happold, F. C. (1939). *Brit. J. exp. Path.* **20**, 396.
Hind, H. G., Lythgoe, B., Macrae, T. F., El Sadr, M. M., Todd, A. R. & Work, C. E. (1939). *Biochem. J.* **33**, 1681.
Hutchings, B. L., Bohonos, N. & Peterson, W. H. (1941). *J. biol. Chem.* **141**, 521.
Kossel, A. (1898). *Hoppe-Seyl. Z.* **25**, 165.
Mueller, J. H. (1937*a*). *J. Bact.* **34**, 163.
—— (1937*b*). *J. Bact.* **34**, 429.
—— & Cohen, S. (1937). *J. Bact.* **34**, 381.
Rinehart, R. E. & West, E. S. (1942). *J. biol. Chem.* **146**, 105.