DISCUSSION

The nature of the violet pigment formed during the reaction is not known. Attempts to isolate the pigment by chromatography and solvent extraction were unsuccessful. It was observed, however, that on addition of mineral acid the violet reaction mixture became orange and this process was reversible. This colour change occurred at pH 9.5–10.1, which may be taken to indicate that the violet product is anionic in nature, possibly involving a keto-enol system.

The foregoing method was developed with the object of estimating combined histidine in the intact protein molecule. With this in view, the proteins chymotrypsin, trypsin, lysozyme, globin, ribonuclease and glucagon were tested, but no colours were obtained. It is particularly surprising that glucagon yielded negative results, as this protein possesses, in its *N*-terminal histidine (Bromer, Sinn, Staub & Behrens, 1956), the structural features apparently requisite for colour production.

SUMMARY

1. A new colorimetric method for the determination of histidine and histamine is described. The method is of use for solutions containing 1–60 µg./ml.

2. The colour reaction is specific for histidine and closely related compounds, namely, histamine and histidine methyl ester. Imidazole does not react and histidine amide gives only a very faint colour.

3. Tryptophan and tyrosine do not form colours under the conditions of the reaction but interfere with histidine colour formation. The interference of some heavy metals and anions is discussed.

The authors would like to express their thanks to Dr O. K. Behrens for a gift of glucagon. Thanks are also due to Dr C. R. Ricketts, of Birmingham Accident Hospital, for gifts of sera and for helpful discussion.

REFERENCES

- Bromer, W. W., Sinn, L. G., Staub, A. & Behrens, O. K. (1956). *J. Amer. chem. Soc.* **78**, 3858.
 Hunter, A. (1952). *J. biol. Chem.* **196**, 589.
 Hunter, A. (1955). *J. biol. Chem.* **216**, 391.
 Jorpes, E. (1932). *Biochem. J.* **26**, 1507.
 Kapeller-Adler, R. (1933). *Biochem. Z.* **264**, 133.
 Knoop, F. (1908). *Beitr. chem. Physiol. Path.* **11**, 356.
 Koessler, K. K. & Hanke, M. T. (1919). *J. biol. Chem.* **39**, 497.
 Macpherson, H. T. (1942). *Biochem. J.* **36**, 59.
 Martell, A. E. & Calvin, M. (1952). In *The Chemistry of the Metal Chelate Compounds*, p. 352. New York: Prentice-Hall.
 Pauly, H. (1904). *Hoppe-Seyl. Z.* **40**, 565.
 Reid, C. & Mulliken, R. S. (1954). *J. Amer. chem. Soc.* **76**, 3869.
 Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 1507.
 Trowbridge, P. F. & Diehl, O. C. (1897). *J. Amer. chem. Soc.* **19**, 588.
 Tsuverkalov, D. A. & Torban, M. A. (1951). *Biokhimiya*, **16**, 74. Quoted in *Chem. Abstr.* (1951). **45**, 5223.
 Voigt, K. (1959). *Biochem. Z.* **331**, 127.
 Zingaro, R. A., VanderWerf, C. A. & Kleinberg, J. (1951). *J. Amer. chem. Soc.* **73**, 88.

Isolation and Structure of a New Factor of the Vitamin B₁₂ Group: Guanosine Diphosphate Factor B

By R. BARCHIELLI, G. BORETTI, A. DI MARCO, P. JULITA,
 A. MIGLIACCI, A. MINGHETTI AND C. SPALLA
Laboratori Ricerche Farmitalia, Milano, Italia

(Received 12 November 1958)

The chromatographic and electrophoretic analysis of the substances having vitamin B₁₂-like activity produced by a strain of *Nocardia rugosa* (Di Marco & Spalla, 1957), and by some mutant strains, indicated the presence of vitamin B₁₂, factor B, and two unknown factors (Di Marco, Alberti, Boretti, Ghione, Migliacci & Spalla, 1957). These two last substances, which we named factors 1 and 2, show a growth-promoting activity only for *Escherichia coli* 113/3 among the vitamin B₁₂-requiring micro-organisms tested, and are charac-

terized by slow mobility in butanol-acetic acid-water (*R_F* values/*R_F* of vitamin B₁₂, 0.12 and 0.3 respectively) (Fig. 1). Factor 1 is electronegative at pH values of 2.7 (Fig. 1) and 6.5. The similarity of the chromatographic and electrophoretic behaviour of these substances to that of factors C₁ and C₂ described by Ford & Porter (1952), Ford, Holdsworth & Kon (1955) and Dellweg, Becher & Bernhauer (1956a) prompted us to attempt the isolation and identification of our factors 1 and 2.

Fig. 1a. Vitamin B₁₂-like factors in *Nocardia rugosa* extracts (N) (mutant strain accumulating factors 1, 2 and B, culture 4 days old). Paper chromatography in *sec.*-butanol-acetic acid-water, electrophoresis in 0.5N-acetic acid, bioautography with *E. coli* 113/3. Markers: vitamin B₁₂ and factor B (5–20 µmg.).

Fig. 1b. Paper electrophoresis of a purified preparation containing factors 1 and 2. I, Without deamination; II, after deamination. Electrophoresis and bioautography are as in Fig. 1a.

Part of the results described in this paper have been reported elsewhere (Barchielli, Boretti, Julita, Migliacci & Minghetti, 1957; Di Marco, Boretti, Migliacci, Julita, Minghetti, 1957).

METHODS

Organisms. The micro-organisms mentioned in the present paper are (1) a strain isolated in our laboratory, designated *Nocardia rugosa*, (2) some mutant strains obtained by irradiation. The characteristics of the species, which are summarized here, are given in detail by Di Marco & Spalla (1957). The mycelium is formed by short hyphae 0.6–0.8 µ in diameter, which radiate from a centre and break up into rods within 30–36 hr. They are Gram-positive and not acid-fast. Good growth is observed after 24–48 hr. incubation at 37° in the usual solid culture media. Growth is colourless, dense and wrinkled. Milk coagulation and gelatin liquefaction are observed. Starch is not hydrolysed. Acids are produced from glucose, D-galactose, D-xylose, rhamnose, L-arabinose, glycerol, D-mannitol and adonitol; nitrates are not reduced.

A culture of the parent strain has been deposited in the National Collection of Industrial Bacteria, England (no. 8926). The mutant strains were obtained by ultraviolet irradiation. The surviving cells were isolated and cultured under the conditions and in the medium described below. The mutants were characterized by their ability to accumulate factors 1, 2 and B, whereas in cultures of the parent strain vitamin B₁₂ predominated.

Growth conditions. Stock cultures were kept on nutrient agar and transferred to liquid medium composed of salt extract (Difco) 40 g., corn steep (Fragda) 5 g., (NH₄)₂SO₄ 5 g., glucose 40 g., N-Z-amine (Sheffield, type E) 15 g., NaCl 10 g., CaCO₃ 5 g., CoCl₂ 10 mg., Tween 80 1 ml. and tap water to 1 l. Fermentations were carried out at 34° in 500 ml. stirred flasks containing 100 ml. of medium or, for extraction purposes, in an 800 l. fermentation vessel containing 500 l. of medium. The highest production of factors is generally obtained after 120–140 hr.

Assay of vitamin B₁₂-like factors. The cultures were examined for the production of vitamin B₁₂ and vitamin B₁₂-like substances. For this purpose assays with *E. coli* 113/3 and *Lactobacillus leichmannii* 7830 were performed in liquid media (Davis & Mingioli, 1950, 1955). The difference between the two titres gives an approximate estimation of the amounts of factors 1, 2 and B present in the culture, as these substances are active as growth factors only for *E. coli* 113/3 (Ghione & Sanfilippo, 1959), whereas *L. leichmannii* responds only to vitamin B₁₂. For example; a culture (120 hr.) of a mutant strain had a growth-promoting activity for *E. coli* (liquid assay) equivalent to 2.86 µg. of vitamin B₁₂/ml. The assay on *L. leichmannii* 7830 showed a 'true vitamin B₁₂' content of 1.6 µg./ml.; therefore the culture contained an amount of factors 1, 2 and B having an activity on *E. coli* equivalent to 1.26 µg. of vitamin B₁₂/ml.

The relative amounts of factors 1, 2 and B may also be roughly evaluated by paper chromatography, followed by bioautography (Winsten & Eigen, 1950) with *E. coli* 113/3.

Extraction and isolation

Fermentation broth (500 l.) of *N. rugosa* (mutant strain producing vitamin B₁₂, factors 1, 2 and B) was supplemented with NaCN (0.5 g./100 ml.) and autoclaved for

30 min. at 120°. After cooling, the broth was treated with 20 g. of Hyflo-Supercel (Johns-Manville & Co.)/l., neutralized to pH 7-7.5 and filtered. The vitamin B₁₂-like factors were adsorbed on charcoal and eluted with propan-2-ol, according to the technique of Dellweg *et al.* (1956*a*). The eluate, concentrated to 6 l., was supplemented with NaCN up to a concentration of 1%. After 4-5 hr. it was saturated with Na₂SO₄ and adjusted to pH 11. The bulk of vitamin B₁₂ was removed by several extractions with benzyl alcohol. The aqueous phase was separated from the alcoholic extracts by centrifuging. During this operation some impurities were precipitated, adsorbing most of factors 1 and 2 and small amounts of vitamin B₁₂ and factor B. This amorphous precipitate, adhering to the centrifuge bowl, was collected and dissolved into 6 l. of water; the solution was adjusted to pH 3 with H₂SO₄ and passed through an Amberlite IRC-50 (H⁺) column (75 mm. inside diameter, 1 m. high) containing 2 l. of resin. The red compounds were then eluted by N-NaOH containing 1% of NaCN. The eluate was extracted with benzyl alcohol as described above. The red precipitate again obtained during extraction, containing about 200 mg. of vitamin B₁₂-like substances (factors 1 and 2, traces of vitamin B₁₂ and factor B), was dissolved in 6 l. of water.

The solution, adjusted to pH 6, was passed through column (inside diameter 15-20 mm., 50 cm. high) of Dowex 1 x 2 (Cl⁻) resin. Factor 1 was selectively adsorbed, and eluted with 5 M-NHCl. The effluent fractions having $E_{276\text{ m}\mu}/E_{367\text{ m}\mu}$ between 0.9 and 0.7 were collected, adjusted to pH 5.5 and evaporated *in vacuo* (40°, 12 mm.) to give a concentration of vitamin B₁₂-like substances of 1.5 mg./ml. The red substances were extracted with phenol ($\frac{1}{3}$ vol.) and precipitated by acetone (about 5 vol.). The vacuum-dried (20°, 12 mm.) precipitate amounted to 150 mg., having a content of total vitamin B₁₂-like factors of about 60% (w/w), as judged colorimetrically. This precipitate was purified by cellulose-column electrophoresis in 0.5 N-acetic acid according to the method of Dellweg, Becher & Bernhauer (1956*b*). The eluted solution of the electro-negative red substance was evaporated under reduced pressure (40°, 12 mm.) and added to 5-10 vol. of acetone. The purity of the red precipitate, dried *in vacuo*, was about 75-80%, measured as before.

Analysis

Guanine. This was usually determined in factor 1 by the colorimetric method of Hitchings (1941), after hydrolysis. The results were checked by the following spectrophotometric method: after removal of the red pigment a fraction of the acid hydrolysate of factor 1 was diluted to 5 ml. with water, then divided into two fractions, one in N-HCl and the other in N-NaOH. $E_{275\text{ m}\mu}$ was determined in both solutions and the guanine content was calculated by comparing the difference between the two values and the data obtained from standard solutions of guanine [$E_{275\text{ m}\mu}$ (NaOH) - $E_{275\text{ m}\mu}$ (HCl) = 0.017/ $\mu\text{g.}$ of guanine/ml.]. The results obtained by the two methods were in good agreement.

Ribose. Ribose was determined by the orcinol reaction of Mejbaum (1939).

Phosphorus. Total phosphorus was determined by the method of Macheboeuf & Delsal (1943); inorganic phosphate was determined by the method of Fiske & Subbarow (1925).

Cobalt. Cobalt was determined according to the method

described by the Istituto Superiore di Sanità (1953) for the determination of cobalt in vitamin B₁₂.

Vitamin B₁₂-like factors. Colorimetric determination of vitamin B₁₂-like substances was carried out by measuring $E_{580\text{ m}\mu}$ of the solution in 0.067 M-Na₂HPO₄ containing 100 $\mu\text{g.}$ of NaCN/ml. The extinction coefficient of vitamin B₁₂ (Friedrich & Bernhauer, 1957*a*) was used for calculating concentrations.

Phosphomonoesterase. This enzyme was prepared from bone according to Gulland & Jackson (1938) (cited by Volkin, 1955).

Paper chromatography and electrophoresis of the vitamin B₁₂-like substances. These were carried out as described by Di Marco, Boretti, Ghione, Migliacci & Sanfilippo (1957).

Detection of guanine in acid hydrolysate of factor 1

About 2 mg. of the preparations containing factors 1 and 2 was dissolved in 2 N-HCl and hydrolysed in a sealed tube for 4 hr. at 110°. After removal of the red pigments by extraction with butanol, the aqueous solution was concentrated and divided into two parts, one of which was deaminated with HNO₃. Paper chromatography performed with two different solvents [butanol-formic acid-water, 77:10:13 (v/v); water adjusted to pH 10 with aq. NH₃] showed that the hydrolysate contained one u.v.-absorbing substance having in each solvent the same R_F as guanine (0.20 and 0.42 respectively). The deaminated hydrolysate contained one u.v.-absorbing compound having the same R_F as xanthine (about 0.6) in water adjusted to pH 10. Determination of the absorption spectra of the spots eluted in 0.1 N-HCl confirmed the identity of the substances with guanine and xanthine respectively.

Enzymic hydrolysis of factor 1

The enzymic hydrolysis of factor 1 was performed by incubation with a crude preparation of *Vipera aspis* venom (Istituto Sieroterapico Milanese) for 3 hr. at 37° at pH 7.6. Preliminary assays had shown that this preparation of viper venom had pyrophosphatase and 5'-nucleotidase activity, but only a slight 3'-nucleotidase activity. After incubation the red pigment was removed by extraction with phenol, precipitated with acetone (6-10 vol.), dried and analysed. The aqueous phase was passed through a column of Dowex 1 x 8 (Cl⁻) resin (0.8 cm. diameter, 3 cm. high) to remove traces of phenol. The filtrate and washings of the column contained a substance having the u.v. spectrum of guanosine (Fig. 3*b*). A total of 3.1 μmoles of guanosine (as determined by spectrophotometry) was obtained from 5.5 μmoles of factor 1.

RESULTS

Identification of factor 1 with guanosine diphosphate factor B

From preliminary examination of the constituents of factors 1 and 2 after separation by electrophoresis on paper, it became clear that factor 1 contained guanine while factor 2 did not; in fact, by treatment with nitrous acid, which converts guanine into xanthine, factor 1 is transformed into a more electronegative growth factor while factor 2 is unchanged (Barchielli *et al.* 1957, confirmed in the present paper, see Fig. 1*b*). Hence it was possible

Table 1. Analyses of three preparations containing mostly factor 1

The values for factor B (determined spectrophotometrically) relate to the combined factor B moiety; there was no free factor B in the preparations. + denotes 'not determined'.

Preparation	...	1	2	3
Factor B (μmoles/mg.)		0.55	0.58	0.55
Factor 1 (%)		+	74	80
Factor 1 (% of factors 1 + 2)		+	87	95
Phosphorus (%)		3.14	3.49	3.57
Ribose (%)		7.59	7.65	7.33
Cobalt (%)		2.88	3.27	+
Guanine (%)		+	7.65	8.25

From these values the following molar ratios per residue of factor B were calculated.

Phosphate	1.83	1.94	2.09
Ribose	0.93	0.87	0.89
Cobalt	0.89	0.96	+
Guanine	+	0.87	0.95

to calculate the exact amount of factor 1 in the preparations from the guanine content. These values are shown in Table 1, together with the detailed analyses carried out on individual preparations.

It may be inferred from the data of Table 1 that factor 1, the main component of our preparations, contains 2 atoms of phosphorus, one molecule of ribose and one of guanine per atom of cobalt.

The absorption spectrum showed that the substances contained in our preparations belong to the incomplete vitamin B₁₂-like factors according to the nomenclature of Friedrich & Bernhauer (1957a). Their aqueous solutions have the absorption spectrum of aquo-complexes: on addition of cyanide the maxima are shifted from 500 and 530 mμ to 540 and 580 mμ, as in dicyano-complexes; maxima at 520 and 550 mμ characteristic of monocyano-complexes were never observed (Fig. 2).

Incubation with *Vipera aspis* venom resulted in the release of guanine riboside, the absorption spectrum of which is very similar to that of 9-substituted derivatives of guanine (Fig. 3).

From these findings we conclude that factor 1 differs from the purine-containing analogues of vitamin B₁₂, since (a) it contains 2 atoms of phosphorus/mol., (b) it does not exist in a form comparable with the monocyano-complexes of the analogues of vitamin B₁₂, and (c) the ribose is linked to the purine at N-9 instead of N-7.

Attempts were made to establish the position of the two phosphate groups. Hydrolysis of factor 1 by *Vipera* venom yielded a red substance, isolated and identified as a monophosphoric acid ester of factor B, and which behaved like factor 2 on paper chromatography. This showed that one of the phosphate groups of factor 1 is linked to the factor B moiety.

Supposing that ribose is present in the furanoside form, as it is in natural ribonucleotides and in other vitamin B₁₂-like substances (Kon & Pawelkiewicz, 1958; Friedrich & Bernhauer, 1956, 1957b), we could assume that the formula of factor 1 corresponded to one of the structures (I), (II), or (III).

Structure (I) was excluded because no acid-labile phosphate is split by heating the substance in N-H₂SO₄ at 100° for 7 min. Structure (II) was also excluded since phosphomonoesterase does not liberate inorganic phosphate from the compound. Dische's carbazole reaction (Dische & Landsberg, 1957) proved that ribose is esterified at C-5; thus two of the alternatives of (III) were ruled out.

We propose, therefore, the alternative of structure (III), involving guanosine 5'-phosphate, as the most likely formula of factor 1. Thus its dicyano-complex would be represented by (IV).

Fig. 2. Absorption spectra of factor 1: ---, in aqueous solutions, without KCN, pH 1-10; —, in 0.067 M-Na₂HPO₄ containing 0.01 % of KCN.

Fig. 3. (a) Absorption spectra of 9-methylguanine (—) and of 7-methylguanine (---) (Gulland & Story, 1938). (b) Absorption spectrum of guanosine liberated from factor 1 by *Vipera* venom.

Fig. 4. Paper chromatography of extracts obtained from *Nocardia rugosa*, cultures 4 days old. I, Strain-accumulating factors 1, 2 and B; Ia, the same, grown in medium with addition of 100 μg . of 5:6-dimethylbenzimidazole/ml.; II, strain-accumulating factor B; IIa, the same, grown in medium with addition of 100 μg . of 5:6-dimethylbenzimidazole/ml.

Comparison was made between guanosine diphosphate factor B (factor 1), from *N. rugosa*, and factors of unknown structure from other sources, by chromatographic and electrophoretic techniques.

E. Lester Smith & E. S. Holdsworth (personal communication) found that our factor 1 was indistinguishable from factor C obtained from *E. coli*. J. Pawelkiewicz (personal communication) observed that factor 1 is very similar to factor y^1 isolated from *Propionibacterium shermanii* (Pawelkiewicz, 1956).

Biological significance of guanosine diphosphate factor B

Both guanosine diphosphate factor B (factor 1) and factor 2, which is probably phosphorylated factor B, are found in cultures of the parent strain of *N. rugosa* in the early stages of growth (24–48 hr.) and disappear later, while more vitamin B_{12} is formed (Di Marco, Alberti, Boretti, Ghione, Migliacci & Spalla, 1957). On the other hand, the cultures of the mutant strain, which has been used for the isolation of factors 1 and 2, contain the factors 1, 2 and B, besides vitamin B_{12} , even after 120 hr. fermentation. On addition of 5:6-dimethylbenzimidazole (100 μg /ml.) at the beginning of growth, only vitamin B_{12} , with sometimes traces of the other factors, is formed [Fig. 4 (I) and (Ia)]. Other unreported experiments showed that if

dimethylbenziminazole is added to the cultures when factors 1 and 2 have already accumulated (for example after 90 hr. fermentation) these disappear rapidly and the vitamin B₁₂ content increases. Another mutant of *N. rugosa* which synthesizes a considerable amount of factor B, traces of factor 1 and 2, and small amounts of vitamin B₁₂, has recently been isolated in our own laboratory (C. Spalla & G. Biffi, unpublished work). The addition of dimethylbenziminazole to cultures of this strain does not increase the formation of vitamin B₁₂, and factor B remains unutilized (Fig. 4).

DISCUSSION

The findings support the hypothesis that factors 1 and 2 are intermediates in the transformation of factor B into vitamin B₁₂ and that their presence results from a deficiency in the biosynthesis of dimethylbenziminazole. This view is strengthened by the likely identity of factor 1 with Pawelkiewicz's factor γ^1 and Ford's factor C, and is in agreement with the opinion of Dellweg *et al.* (1956*a*) on the biological significance of factor C, and with some observations of Ghione & Sanfilippo (1959) on the utilization of factor 1 by *E. coli*.

It may be recalled that a coenzymic role has been recently ascribed to the nucleotides of guanine in some reactions. Munch-Petersen (1955) described the following pyrophosphorolytic reaction: guanosine diphosphomannose + pyrophosphate $\rightarrow$ guanosine triphosphate + mannose phosphate. In a similar way the phosphorolysis of guanosine diphosphate factor B could lead to the formation of phosphorylated factor B, which might be the immediate precursor of vitamin B₁₂. It is probable that our factor 2 is such a phosphorylated factor B.

The reaction cytidine diphosphate choline + diglyceride $\rightarrow$ lecithin + cytidylic acid (Kennedy, 1957) suggests that guanosine diphosphate factor B may play the role of intermediate in the following reactions: factor B + adenosine triphosphate $\rightarrow$ phosphorylated factor B; phosphorylated factor B + guanosine triphosphate $\rightarrow$ pyrophosphate + guanosine diphosphate factor B; guanosine diphosphate factor B + dimethylbenzoxymaline riboside $\rightarrow$ vitamin B₁₂ + guanylic acid.

SUMMARY

1. A substance of the vitamin B₁₂ group, identified as guanosine diphosphate factor B, has been isolated from cultures of a strain of *Nocardia rugosa*.

2. Evidence is submitted which indicates that this substance (factor 1) is a guanosine 5'-pyrophos-

phoric ester of factor B, in which ribose is linked to N-9 of guanine.

3. The significance of this compound as a possible intermediate in the biosynthesis of vitamin B₁₂ is discussed.

REFERENCES

- Barchielli, R., Boretti, G., Julita, P., Migliacci, A. & Minghetti, A. (1957). *Biochim. biophys. Acta*, **25**, 452.
 Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
 Davis, B. D. & Mingioli, E. S. (1955). U.S. Patent xv, p. 885.
 Dellweg, H., Becher, E. & Bernhauer, K. (1956*a*). *Biochem. Z.* **328**, 81.
 Dellweg, H., Becher, E. & Bernhauer, K. (1956*b*). *Biochem. Z.* **328**, 88.
 Di Marco, A., Alberti, G. C., Boretti, G., Ghione, M., Migliacci A. & Spalla, C. (1957). *Europäisches Symposium über vitamin B₁₂ und Intrinsic Factor*, p. 55. Edited by H. C. Heinrich. Stuttgart: Enke Verlag.
 Di Marco, A., Boretti, G., Ghione, M., Migliacci, A. & Sanfilippo, A. (1957). *G. Biochim.* **6**, 5, 219.
 Di Marco, A., Boretti, G., Migliacci, A., Julita, P. & Minghetti, A. (1957). *Boll. Soc. ital. Biol. sper.* **33**, 1513.
 Di Marco, A. & Spalla, C. (1957). *G. Microbiol.* **4**, 24.
 Dische, Z. & Landsberg, E. (1957). *Biochim. biophys. Acta*, **24**, 193.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Ford, J. E., Holdsworth, E. S. & Kon, S. K. (1955). *Biochem. J.* **59**, 86.
 Ford, J. E. & Porter, J. W. G. (1952). *Biochem. J.* **51**, 5.
 Friedrich, W. & Bernhauer, K. (1956). *Angew. Chem.* **68**, 580.
 Friedrich, W. & Bernhauer, K. (1957*a*). In *Biochemisches Taschenbuch*, p. 478. Ed. by Rauen, H. M. Berlin: Springer Verlag.
 Friedrich, W. & Bernhauer, K. (1957*b*). *Chem. Ber.* **90**, 1966.
 Ghione, M. & Sanfilippo, A. (1959). *G. Microbiol.* **6**, 113.
 Gulland, J. M. & Jackson, E. M. (1938). *Biochem. J.* **32**, 590.
 Gulland, J. M. & Story, L. F. (1938). *J. chem. Soc.* p. 693.
 Hitchings, G. H. (1941). *J. biol. Chem.* **139**, 843.
 Kennedy, E. P. (1957). *Annu. Rev. Biochem.* **26**, 134.
 Kon, S. K. & Pawelkiewicz, J. (1958). *4th int. Congr. Biochem., Vienna*, Symposium 11.
 Istituto Superiore di Sanità, Roma (1953). *Metodi di controllo delle vitamine*.
 Macheboeuf, M. A. & Delsal, J. (1943). *Bull. Soc. chim. Biol.* **25**, 116.
 Mejbbaum, W. (1939). *Hoppe Seyl. Z.* **258**, 117.
 Munch-Petersen, A. (1955). *Arch. Biochem. Biophys.* **55**, 592.
 Pawelkiewicz, J. (1956). *Acta biochim. polon.* **3**, 581.
 Volkin, E. (1955). In *Methods in Enzymology*, vol. 2, p. 539. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Winsten, W. A. & Eigen, E. (1950). *J. biol. Chem.* **184**, 155.