- Loening, U. E. (1960). *Biochem. J.* **77**, 20r.
- Lund, H. A., Vatter, A. E. & Hanson, J. B. (1958). *J. biophys. biochem. Cytol.* **4**, 87.
- Palade, G. E. & Siekevitz, P. (1956). *J. biophys. biochem. Cytol.* **2**, 171, 671.
- Peterman, M. L., Hamilton, M. G., Balis, M. E., Samarth, K. & Pecora, P. (1958). In *Microsomal Particles and Protein Synthesis*, p. 70. Ed. by Roberts, R. B. London: Pergamon Press Ltd.
- Slater, T. F. (1958). *Biochim. biophys. Acta*, **27**, 201.
- Ts'o, P. O. P., Bonner, J. & Vinograd, J. (1956). *J. biophys. biochem. Cytol.* **2**, 452.
- Ts'o, P. O. P. & Sato, C. S. (1959). *Exp. Cell Res.* **17**, 227.
- Wakid, N. W. (1960). *Biochem. J.* **76**, 88.
- Webster, G. C. (1957). *J. biol. Chem.* **229**, 535.
- Whaley, W. G., Mollenhauer, H. H. & Leech, J. H. (1960). *Amer. J. Bot.* **47**, 401.

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New Pigments Derived from Vitamin B₁₂ by a Strain of *Aerobacter aerogenes* Present in River Mud

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Micro-organisms have proved very useful in producing not only vitamin B₁₂, but also vitamin B₁₂ analogues and some of their derivatives. Thus a number of vitamin B₁₂ analogues containing either purines or benzimidazoles have been prepared by guided biosynthesis with a vitamin-B₁₂-requiring mutant of *Escherichia coli* (Ford, Holdsworth & Kon, 1955; Bernhauer & Friedrich, 1954). As a result of microbial activity a number of vitamin B₁₂ analogues have been isolated from the gut and rumen contents, faeces of ruminants (Kon, 1955; Porter, 1957; Dion, Calkins & Pfiffner, 1952; Lewis, Tappan & Elvehjem, 1952a, b; Brown, Cain, Gant, Parker & Smith, 1955) and from sewage sludge (Friedrich & Bernhauer, 1953; Neujahr, 1956).

Phosphorylated factor B and guanosine diphosphate factor B have been isolated from cultures of *Nocardia rugosa* (Barchielli *et al.* 1960). A number of intermediates in the biosynthesis of vitamin B₁₂ have been isolated from *Propionibacterium shermanii* (Bernhauer, Becher, Gross & Wilharm, 1960); these include nucleotide-free carboxylic acids (amides of cobinic acid) and vitamin B₁₂ carboxylic acids. A new group of coenzymes, which are derivatives of vitamin B₁₂ or certain analogues, have been prepared from *Clostridium tetanomorphum* (Barker *et al.* 1960a). The vitamin B₁₂ coenzyme has also been prepared from *P. shermanii* (Barker *et al.* 1960b).

Compared with the work quoted above surprisingly little has been reported on the action of micro-organisms on vitamin B₁₂ and its analogues. Ford & Porter (1953) have shown that organisms present in calf faeces will induce interconversion between analogues, and micro-organisms from

rotten fish were shown to degrade vitamin B₁₂ to unidentified products (Mori, Hashimoto & Malda, 1953, 1954; Mori & Malda, 1955). In the present work the action of *Aerobacter aerogenes* on vitamin B₁₂ is described.

A preliminary account of some of this work has already been given (Helgeland, Jonsen & Laland, 1959).

MATERIALS AND METHODS

Vitamin B₁₂. This was a gift from Glaxo Laboratories Ltd., Greenford, Middlesex.

[⁵⁸Co]Vitamin B₁₂. this was purchased from The Radiochemical Centre, Amersham, Bucks., and had a specific activity of 0.22 µCi/µg.

Media. The synthetic medium contained: NH₄Cl, 2 g.; Na₂HPO₄·2H₂O, 6 g.; KH₂PO₄, 3 g.; NaCl, 3 g.; MgCl₂·6H₂O, 0.04 g.; Na₂SO₄, 0.14 g.; water to 900 ml. The pH was adjusted to 7.2 with 10% NaOH before autoclaving. Depending on the experimental conditions, to 9 vol. of media was added either (a) 1 vol. of sterile water or (b) 1 vol. of a boiled solution containing glucose and vitamin B₁₂ or vitamin B₁₂ only.

The meat-extract medium contained: beef extract (Difco), 1.5 g.; peptone (Difco) 5 g.; Na₂HPO₄·2H₂O, 1 g.; water to a total volume of 500 ml. The pH of the solution was adjusted to 7.8 with 10% NaOH before autoclaving. When required, 0.1 mg. of vitamin B₁₂/ml. was added before autoclaving.

The agar medium contained: meat extract (Difco), 9 g.; peptone (Difco), 30 g.; Na₂HPO₄·2H₂O, 6.76 g.; NaCl, 9 g.; water to 1500 ml. The pH of the solution was adjusted to 8.8 with 10% NaOH and agar (37.5 g.) was added before autoclaving.

Subculturing of micro-organism obtained from river mud. A portion (0.1 ml.) of a suspension in 0.9% NaCl solution of mud obtained from a stream in the suburbs of Oslo was used to inoculate 3 ml. of the synthetic medium containing 0.34% (w/v) of glucose and 0.3 mg. of vitamin B₁₂.

Incubation was carried out in a test tube (1 cm. × 10 cm.) for 5 days at 37°. The micro-organisms were subcultured in a medium and in a manner identical with that described above.

Growth of other bacteria in the presence of vitamin B₁₂. The medium used consisted of: FeSO₄·7H₂O, 0.75 mg.; CuSO₄·5H₂O, 7.5 mg.; ZnSO₄·7H₂O, 7.5 mg.; MnSO₄·4H₂O, 75 mg.; CaCl₂·2H₂O, 37.5 mg.; MgSO₄, 0.3 g.; KH₂PO₄, 0.75 g.; (NH₄)₂SO₄, 3 g.; yeast extract (Difco), 3 g.; glucose, 0.75 g.; water to 1.5 l. Test tubes (1 cm. × 10 cm.) containing 3 ml. of medium were inoculated with one of the following bacteria, which were freshly isolated laboratory strains: *Bacillus subtilis*, *B. cereus*, *Streptococcus faecalis*, *Sarcina lutea* or *Staphylococcus aureus* H. The tubes were incubated for 12 days at 37°.

Bacterial growth. This was measured in a Beckman spectrophotometer at 650 mμ.

Extraction of incubation mixtures. Unchanged vitamin B₁₂ and pigments derived from the vitamin were extracted from the medium after removal of the cells by centrifuging, by brief shaking with 0.5 g. of phenol (Laland & Klem, 1936). The phenol layer, which contained all the colour, was removed after centrifuging the mixture and washed twice with 3 vol. of water to remove salts (a procedure which did not remove any colour). To the phenol layer was then added 6 vol. of ether and 1 vol. of water. After mixing and separation of the two phases by centrifuging, the coloured aqueous layer was washed twice with 2 vol. of ether and freeze-dried, and the solids were dissolved in an appropriate amount of water for further purification.

Paper chromatography. Descending chromatography on Whatman no. 1 paper at 25° was used. The solvent systems were butan-2-ol saturated with water-acetic acid-KCN (9.9:0.1, v/v, with 10 mg. of KCN/100 ml.) and butan-2-ol saturated with water-NH₃ solution (sp.gr. 0.88)-KCN (9.9:0.1, v/v, with 10 mg. of KCN/100 ml.) (Kon, 1955).

Paper electrophoresis. The apparatus described by Durrum (1951) was used with a voltage gradient of 10–12 v/cm., usually applied for 5–7 hr. on Whatman no. 1 or no. 3 MM paper. The following buffers were used: (1) n-acetic acid containing 0.01% of KCN, pH about 2; (2) 0.05 M-phosphate (a mixture of KH₂PO₄ and K₂HPO₄) containing 0.01% of KCN, pH 6.5; (3) 0.1 M-KCN, pH about 10.

Absorption spectra. These were determined in water and in 0.1 M-KCN in a Beckman spectrophotometer with photomultiplier.

Measurement of radioactivity. The activity of radioactive paper strips was measured by cutting out 1 cm. × 2 cm.

pieces and counting these on aluminium disks in a thin end-window Geiger-Müller counter.

Radioactive solutions were plated and counted on aluminium disks at infinite thinness with a Tracerlab flow counter. Counting was continued until the count was 3000.

RESULTS

Microbiological preparation of pigments derived from vitamin B₁₂: their electrophoretic and chromatographic properties

Five tubes as shown in Table 1 were inoculated with a suspension in 0.9% NaCl solution of mud obtained from a stream. Phenol-extractable material from the incubation mixtures was examined by paper electrophoresis in acetic acid and by paper chromatography in butan-2-ol saturated with water-acetic acid-KCN. On electrophoresis, the extracts from tubes no. 1 and 4 gave four coloured zones, which moved towards the cathode. These were aquocobalamin, a brownish-yellow pigment (pigment I), a yellow pigment (pigment II) and unchanged vitamin B₁₂. Pigments I and II had mobilities 0.78 and 0.33 respectively relative to that of aquocobalamin. Paper chromatography of the same extracts gave aquocobalamin, *R_F* 0.09, unchanged vitamin B₁₂, *R_F* 0.25, a brownish-yellow pigment (pigment I), *R_F* 0.35, and a yellow pigment (pigment II), *R_F* 0.39.

The pigments I and II were not formed when vitamin B₁₂ was omitted (Table 1).

Pigments I and II isolated by direct paper chromatography of the extracts corresponded, when run electrophoretically, to the similarly coloured spots obtained by direct electrophoresis of the extracts.

The presence of aquocobalamin on the electrophoretic and chromatographic strips was shown by its spectrum in water (Veer, Edelhausen, Wijmenga & Lens, 1950). Further, 0.1 M-KCN converted it into vitamin B₁₂, as indicated by spectral and chromatographic properties.

When extract from tube no. 1 (Table 1) was run electrophoretically in phosphate buffer at pH 6.5, a negatively charged brownish-yellow zone (mobility

Table 1. *Conditions for formation of pigments I and II*

Tubes (1 cm. × 10 cm.) containing 3 ml. of medium were inoculated with 0.05 ml. of a suspension in 0.9% NaCl solution of mud from a stream and incubated for 5 days at 37°. The presence of pigments I and II in the material extractable with phenol from cell-free incubation mixture were detected after paper electrophoresis in n-acetic acid.

Tube no.	Medium	Glucose (% w/v)	Vitamin B ₁₂ (mg.)	Bacterial growth	Formation of pigments I and II
1	Synthetic	0.34	0.3	+	+
2	Synthetic	0.34	0	+	—
3	Synthetic	0	0.3	—	—
4	Beef extract-peptone	0	0.3	+	+
5	Beef extract-peptone	0	0	+	—

0.85 relative to aquocobalamin), which separated into pigments I and II when run in butan-2-ol saturated with water-acetic acid-KCN, was obtained. In addition aquocobalamin with one positive charge, unchanged vitamin B₁₂ with no charge, and a pink negatively charged zone were detected. This latter unknown zone was not further investigated.

In 0.1 M-KCN pigments I and II had the same mobility as vitamin B₁₂.

Absorption spectra

Pigments I and II were purified by repeated (twice) paper chromatography in butan-2-ol saturated with water-acetic acid-KCN followed by paper electrophoresis in acetic acid. The two substances purified in this manner were stored in the freeze-dried state.

In recording the spectra (Fig. 1) for pigment I, molecular extinction coefficients are used. These could, as shown later, be calculated by using [⁵⁸Co]vitamin B₁₂ for the preparation of pigment I. For pigment II, extinction values only are recorded (Fig. 2).

In Fig. 1 a comparison is made between the spectra of pigment I and vitamin B₁₂ in water. Pigment I exhibits a distinct peak in the ultra-violet region at 262 mμ, somewhat displaced towards lower wavelength as compared with the vitamin (278 mμ). The most conspicuous feature is, however, the small peak at 342 mμ as compared with the large peak in the 300–400 mμ region for

vitamin B₁₂. For pigment I the peak in the visible region occurs at 474 mμ.

When pigment I (brownish-yellow) was dissolved in 0.1 M-KCN, the solution turned pink with a pronounced bathochromic shift of the maximum from 474 to 500 mμ (Fig. 1). This property is similar to that of vitamin B₁₂, which also exhibits a bathochromic shift in KCN with a concomitant change in colour. On acidifying with acetic acid the colour changed back to brownish-yellow and pigment I was reisolated by paper chromatography and paper electrophoresis.

The noticeable feature in the spectrum of pigment II (Fig. 2) in water is the absence of a large peak in the 300–400 mμ region. When dissolved in 0.1 M-KCN there was a bathochromic shift in the maximum from 448 to 482 mμ.

The bathochromic shift in the spectra of pigments I and II in KCN was probably due to complex-formation (as with vitamin B₁₂) with cyanide and not merely to a change in pH, since no such shift was experienced when to aqueous solutions of pigments I and II was added NH₃ to the same pH (10).

Presence of cobalt in pigment I

Synthetic medium (3 ml.) containing 0.34 % of glucose, 1.2 μg. of [⁵⁸Co]vitamin B₁₂ (6920 counts/min.) and 0.3 mg. of inactive vitamin B₁₂ was inoculated with organisms subcultured five times in the synthetic medium containing glucose and vitamin B₁₂. The radioactive culture was incubated for 5 days at 37°, and pigment I isolated from the phenol-extractable material by paper electrophoresis in acetic acid and further purified by

Fig. 1. Absorption spectra of vitamin B₁₂ in water (—), and pigment I in water (---) and in 0.1 M-KCN (— · —).

Fig. 2. Absorption spectra of pigment II in water (—), and in 0.1 M-KCN (---).

paper chromatography in butan-2-ol saturated with water-acetic acid-KCN. The radioactivity along the strip was counted. At this stage of purification some vitamin B₁₂ was present and probably originated from contaminating aquo-cobalamin, which runs close to pigment I in the initial electrophoretic separation. The radioactive area containing pigment I was purified further by paper chromatography in butan-2-ol saturated with water-NH₃ solution-KCN. To exclude the possibility that some radioactive vitamin B₁₂ was still only loosely adsorbed to pigment I, 0.05 mg. of inactive vitamin B₁₂ was mixed with the eluted radioactive pigment I and the mixture submitted to electrophoresis in acetic acid. Examination of the electrophoretic strip revealed that pigment I retained its radioactivity and therefore contains cobalt.

Molecular extinction coefficient of pigment I

An incubation mixture similar to that described above was prepared in duplicate. By using the light absorption at 548 m μ to calculate the amount of vitamin B₁₂ present, the average specific activity of vitamin B₁₂ in the two solutions was determined before incubation (65.5 counts/min./ μ g.). The phenol-extractable material obtained after incubation from the two incubation mixtures was purified by electrophoresis in N-acetic acid. Pigment I was subsequently purified by paper chromatography in butan-2-ol saturated with water-acetic acid-KCN, eluted and freeze-dried. The material was dissolved in 1 ml. and the absorption at 474 m μ determined; 0.1 ml. was used for radioactivity measurements. Assuming one atom of cobalt per molecule the molecular extinction coefficient was calculated for the two duplicates (7.9×10^3 and 8.8×10^3 respectively). The remaining material from the duplicates was combined and run in butan-2-ol saturated with water-NH₃ solution-KCN. Pigment I was eluted and the molecular extinction coefficient again determined (8.5×10^3). The mean molecular extinction coefficient at 474 m μ is therefore 8.4×10^3 and was used for the calculations to be described.

Formation of pigment I under different conditions

An extract containing pigment I was obtained from the incubation mixtures by the phenol method. The pigment was separated from other material by electrophoresis in N-acetic acid. The zone of pigment I was eluted from the paper with 3 ml. of water and the absorption at 474 m μ used to calculate the amount of pigment present.

Varying amounts of vitamin B₁₂. Each tube contained 3 ml. of synthetic medium with 0.34 % (w/v) of glucose and vitamin B₁₂ and was incubated for 5 days at 37° after inoculation with micro-

organisms subcultured seven times. There was a linear relationship between the amount of vitamin B₁₂ per tube and pigment I produced. The maximum amount of vitamin B₁₂ per tube tested was 660 μ m-moles, which gave 20 μ m-moles of pigment I.

Incubation time. The relationship between the formation of pigment I and the incubation period is shown in Fig. 3. The production of pigment I took place at a much slower rate than the bacterial growth. To obtain a maximum yield of pigment I, an incubation period of at least 12 days was required.

Effect of glucose concentration. The yield of pigment I was very dependent on the glucose concentration (Fig. 4). The highest yield was obtained

Fig. 3. Time curves for the formation of pigment I (—) and the bacterial growth (----). Each tube contained 3 ml. of synthetic medium with 0.34 % (w/v) of glucose and 200 μ m-moles of vitamin B₁₂. The tubes were incubated at 37° after inoculation with micro-organisms subcultured seven times. The amount of pigment I formed was determined as described in the text.

Fig. 4. Formation of pigment I (—) at different concentrations of glucose. The bacterial growth curve (----) is also recorded. Each tube contained 3 ml. of synthetic medium with 0.3 mg. of vitamin B₁₂ and glucose and were incubated for 5 days at 37° after inoculation with micro-organisms subcultured seven times. The amount of pigment I formed was determined as described in the text.

Table 2. *Effect of oxygen on the yield of pigment I*

Each incubation mixture consisted of 3 ml. of synthetic medium containing 0.8% (w/v) of glucose and 780 μ -moles of vitamin B₁₂. After inoculation with micro-organisms subcultured seven times, the mixtures were incubated at 37° for 12 days. Aerobic incubation was carried out with occasional shaking in a 10 ml. conical flask (surface area of incubation mixture 7 cm.²). Anaerobic incubation was in a test tube (1 cm. x 10 cm.) in an atmosphere of hydrogen, and the partly anaerobic incubation in the same kind of test tube in the presence of oxygen (without shaking). The amount of pigment I formed was determined as described in the text.

Conditions	Amount of pigment I formed (μ -moles)	$E_{450\text{ m}\mu}$ of culture
Aerobic	4.6	0.52
Anaerobic	20.6	0.18
Partly anaerobic	19.2	0.42

with a glucose concentration in the range 0.8–1.2%. It is also seen that a large bacterial growth does not necessarily produce a better yield of pigment I.

Aerobic and anaerobic formation of pigment I. Pigment I was produced both under aerobic and anaerobic conditions, but anaerobic conditions greatly favour its formation (Table 2).

Identification of micro-organism

By subcultivation of the incubation mixtures on agar plates, rough and smooth colonies of *A. aerogenes* were obtained. Transformation of vitamin B₁₂ was subsequently achieved by subcultures from single colonies of both the rough- and the smooth-colony type.

Formation of pigment I by other bacteria

None of the following bacteria, *B. subtilis*, *B. cereus*, *Streptococcus faecalis*, *Sarcina lutea* and *Staphylococcus aureus* (Strain H), when grown on a glucose plus salts medium containing vitamin B₁₂ produced detectable quantities of pigment I as judged by paper electrophoresis in acetic acid of phenol-extractable material of the cell-free incubation mixture.

DISCUSSION

The absorption spectrum of the brownish-yellow pigment I derived from vitamin B₁₂ exhibits a distinct peak in the ultraviolet region (Fig. 1), suggesting the presence of the nucleotide in the molecule. The absence of a large peak in the 300–400 $m\mu$ region in pigment I as compared with vitamin B₁₂ is also found in other vitamin B₁₂ derivatives such as Beaven & Johnson's (1955) brownish reduction product, breakdown products of vitamin B₁₂ produced as a result of γ -irradiation

(Sjöstedt & Ericson, 1959), the cobamide coenzymes (Barker *et al.* 1960*a, b*), the product(s) obtained when reducing aquocobalamin catalytically in the presence of adenine (Bernhauer, Gaiser, Müller & Wagner, 1960), the solution of cobinamide reduced with sodium dithionite in the presence of adenine (Pawelkiewicz, Bartosinski & Walerych, 1960) and the solution of factor B treated with the same reducing agent (George, Irvine & Glauser, 1960). Pigment I, however, is stable towards air and daylight and is therefore different from these products.

The yield of pigment I is considerably higher under anaerobic conditions and it is therefore possible that some reduction of vitamin B₁₂ is involved in its formation. Several reduction products of vitamin B₁₂ involving the central cobalt atom are described in the literature. On catalytic hydrogenation a brownish-coloured reduction product of vitamin B₁₂ (Kaczka, Wolf & Folkers, 1949; Diehl & Murie, 1952; Boos, Carr & Conn, 1953; Beaven & Johnson, 1955) containing bivalent cobalt, as shown by polarographic and titrimetric evidence (Jasalkis & Diehl, 1954), is obtained. This substance is readily reoxidized to hydroxocobalamin by exposure to air. A second, more highly reduced, grey-green substance is also reoxidizable to hydroxocobalamin (Diehl & Murie, 1952; Boos *et al.* 1953; Beaven & Johnson, 1955).

A brownish-green colour is produced when vitamin B₁₂ is heated with alkali in the absence of air. It has been shown that a reduction in the valency of the cobalt atom accompanied by oxidation in the B-ring and its propionamide side chain has occurred (Smith, 1955; Bonnett *et al.* 1957). In this case also the cobalt atom is reoxidized upon exposure to air as shown by the return of the red colour.

The cobamide coenzymes have recently been shown to contain bivalent cobalt (Bernhauer, Gaiser, Müller, Müller & Günter, 1960; Johnson & Shaw, 1960) and are, in contrast with the reduction products described above, stable to oxygen provided no exposure to light occurs. The presence in the coenzymes of the adenine nucleoside somehow stabilizes the bivalent cobalt co-ordination complex.

Pigment I might have a structure analogous to the coenzymes on the basis of its absorption spectrum and its favoured formation under anaerobic conditions. The increased stability of pigment I in daylight under oxygen and towards potassium cyanide as compared with the coenzymes could be due to the nature of the substance which is co-ordinating with the bivalent cobalt in pigment I.

Another possible feature of the structure for pigment I, which could account for its spectrum, would be a molecule in which some interruption in the six conjugated double bonds present in vitamin B₁₂ had occurred.

To explain the formation of pigment I one also must take into account its positive charge in N-acetic acid-potassium cyanide (pH 2.5), and its negative charge in phosphate buffer (pH 6.5) and potassium cyanide (pH 10).

It is tentatively suggested that the formation of pigment I by *A. aerogenes* involves the reduction of the cobalt atom in vitamin B₁₂ followed by co-ordination of the bivalent cobalt to some ionizable substance provided by the micro-organism. The reaction could be enzymic or non-enzymic.

SUMMARY

1. Crude cultures from river mud containing predominantly *Aerobacter aerogenes* produces a brownish-yellow pigment (pigment I) and a yellow pigment (pigment II) when grown in the presence of vitamin B₁₂ in a synthetic salt-glucose medium or a meat-extract medium. Pure cultures of both the rough and the smooth type of *Aerobacter aerogenes* were also shown to possess this property.

2. Both pigments are derived from vitamin B₁₂. Pigment I has been shown to contain cobalt. It is positively charged at pH 2.5 and negatively charged at 6.5. It has a distinct peak in the ultraviolet region at 262 mμ and lacks the large peak in the 300–400 mμ region characteristic of vitamin B₁₂. In potassium cyanide the pigment turns pink and there is a bathochromic shift in the spectrum. This reaction is reversible. Pigment II is also positively charged at pH 2.5 and negatively charged at pH 6.5 and lacks the large peak in the 300–400 mμ region.

3. Within certain limits the formation of pigment I increases linearly with the concentration of vitamin B₁₂. The formation of this substance is very slow and requires 12 days to obtain maximum yield. Anaerobic conditions greatly favour its formation, and the yield is also very dependent on the glucose concentration, reaching a maximum at a glucose concentration in the region 0.8–1.2%.

4. No detectable formation of pigment I and II was achieved with the following bacteria: *Bacillus subtilis*, *B. cereus*, *Streptococcus faecalis*, *Sarcina lutea* and *Staphylococcus aureus* H.

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REFERENCES

- Barchielli, R., Boretti, G., Di Marco, A., Julita, P., Migliacca, A., Minghetti, A. & Spalla, C. (1960). *Biochem. J.* **74**, 382.
- Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Petersen, A., Toohey, J. I., Ladd, J. N., Volcani, B. E. & Wilson, R. M. (1960a). *J. biol. Chem.* **235**, 181.
- Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N. & Volcani, B. E. (1960b). *J. biol. Chem.* **235**, 480.
- Beaven, G. H. & Johnson, E. A. (1955). *Nature, Lond.*, **176**, 1264.
- Bernhauer, K., Becher, E., Gross, G. & Wilharm, G. (1960). *Biochem. Z.* **332**, 562.
- Bernhauer, K. & Friedrich, W. (1954). *Angew. Chem.* **66**, 776.
- Bernhauer, K., Gaiser, P., Müller, O., Müller, E. & Günter, F. (1960). *Biochem. Z.* **333**, 560.
- Bernhauer, K., Gaiser, P., Müller, O. & Wagner, O. (1960). *Biochem. Z.* **333**, 106.
- Bonnett, R., Cannon, J. R., Clark, V. M., Johnson, A. W., Parker, L. F. J., Smith, E. L. & Todd, A. R. (1957). *J. chem. Soc.* p. 1158.
- Boos, R. N., Carr, J. E. & Conn, J. B. (1953). *Science*, **117**, 603.
- Brown, F. B., Cain, J. C., Gant, D. E., Parker, L. F. J. & Smith, E. L. (1955). *Biochem. J.* **59**, 82.
- Diehl, H. & Murie, R. (1952). *Iowa St. Coll. J. Sci.* **26**, 555.
- Dion, H. W., Calkins, D. G. & Pfiffner, J. J. (1952). *J. Amer. chem. Soc.* **74**, 1108.
- Durrum, E. L. (1951). *Science*, **113**, 66.
- Ford, J. E., Holdsworth, E. S. & Kon, S. K. (1955). *Biochem. J.* **59**, 86.
- Ford, J. E. & Porter, J. W. G. (1953). *Brit. J. Nutr.* **7**, 326.
- Friedrich, W. & Bernhauer, K. (1953). *Angew. Chem.* **65**, 627.
- George, P., Irvine, D. H. & Glauser, S. C. (1960). *Ann. N.Y. Acad. Sci.* **88**, 393.
- Helgeland, K., Jonsen, J. & Laland, S. (1959). *Acta chem. scand.* **13**, 2128.
- Jasalkis, B. & Diehl, H. (1954). *J. Amer. chem. Soc.* **76**, 4345.
- Johnson, A. W. & Shaw, N. (1960). *Proc. chem. Soc., Lond.*, p. 420.
- Kaczka, A., Wolf, D. E. & Folkers, K. (1949). *J. Amer. chem. Soc.* **71**, 1514.
- Kon, S. K. (1955). *Symp. biochem. Soc.* **13**, 17.
- Laland, P. & Klem, Å. (1936). *Acta med. scand.* **88**, 620.
- Lewis, U. J., Tappan, D. V. & Elvehjem, C. A. (1952a). *J. biol. Chem.* **194**, 539.
- Lewis, U. J., Tappan, D. V. & Elvehjem, C. A. (1952b). *J. biol. Chem.* **199**, 517.
- Mori, T., Hashimoto, Y. & Malda, Y. (1953). *Bull. Jap. Soc. sci. Fish.* **19**, 135.
- Mori, T., Hashimoto, Y. & Malda, Y. (1954). *Bull. Jap. Soc. sci. Fish.* **20**, 604.
- Mori, T. & Malda, Y. (1955). *Bull. Jap. Soc. sci. Fish.* **21**, 492.
- Neujahr, H. Y. (1956). *Acta chem. scand.* **10**, 917.
- Pawelkiewicz, J., Bartosinski, B. & Walerych, W. (1960). *Bull. Acad. polon. Sci.* **8**, 123.
- Porter, J. W. G. (1957). *Vitamin B₁₂ and Intrinsic Factor*, 1. *Europäisches Symposium, Hamburg* (1956), p. 43. Ed. by Heinrich, H. G. Stuttgart: Ferdinand Enke.
- Sjöstedt, M. & Ericson, L. E. (1959). *Acta chem. scand.* **13**, 1711.
- Smith, E. L. (1955). *Symp. biochem. Soc.* **13**, 3.
- Veer, W. L. C., Edelhausen, J. H., Wijmenga, H. G. & Lens, J. (1950). *Biochim. biophys. Acta*, **6**, 225.