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Enzymatic decyanation of cyanocobalamin in rat tissues

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Cyanocobalamin is decyanated *in vitro* to hydroxocobalamin by rat liver and kidney. This appears to be due to an enzyme system which we have called "cyanocobalamin-decyanase". The enzyme is in the soluble fraction of the cells; it requires reduced cozymases and flavins and has an optimum pH of 7·2 under anaerobic conditions. This is consistent with the fact that conversion of cyanocobalamin to hydroxocobalamin and/or its reduced forms ($B_{12}r$, $B_{12}s$) involves a reductive process in the earliest stage of the biosynthesis of coenzyme B_{12}.

VITAMIN B_{12} coenzyme (5′-deoxyadenosyl-B_{12}, DBC-coenzyme) accounts for as much as 72% of cobamide compounds in animal liver (Toohey & Barker, 1961). Since it is less stable than the cobamide vitamins, these authors felt that the true percentage in the liver may be even higher and previous failures in its isolation may be due to its lability.

Although the presence of small amounts of hydroxocobalamin and cyanocobalamin in liver has not yet been excluded, most of the hydroxocobalamin found as "native" vitamin B_{12} very likely arises from the cleavage of the coenzyme or cyanocobalamin or both.

Structural assignments based upon the cleavage of the coenzyme by photolysis or acid hydrolysis or both, demonstrated that the coenzyme possesses all the structural features of cyanocobalamin; in the former the deoxyadenosyl moiety takes the place of the cyano-group (Smith, Mervyn, Johnson & Shaw, 1962).

The chemical pathway to the coenzyme (and probably also the enzymatic one) proceeds *via* a two-electron reduction of cyano- or hydroxocobalamin in presence of air (Johnson, Mervyn, Shaw & Smith, 1963; Smith, Mervyn, Muggleton, Johnson & Shaw, 1964; Peterkofsky & Weissbach, 1964).

Hydroxocobalamin seems therefore to play a primary role both in the cleavage of the coenzyme (Brady & Barker, 1961; Hogenkamp, 1964) and in its biosynthesis from cyanocobalamin (Fenrych, Pawelkiewicz & Magas, 1962; Górna, 1963; Pawelkiewicz, Górna, Fenrych & Magas, 1964).

We set out to elucidate the early metabolic step in the biosynthesis of the coenzyme B_{12} from cyanocobalamin. We describe the enzymatic conversion of cyano- to hydroxocobalamin in tissues of high cobalamin turnover, like liver and kidney, and discuss the different roles of these tissues in the metabolism and storage of the two cobalamins (Cima, Mastrogiacomo & Maraschin, 1965; Mastrogiacomo, Cima & Maraschin, 1965).

Experimental

MATERIALS AND METHODS

Preparation of tissues samples. All tissues samples were prepared at 0 to 5°. Male albino rats (Wistar) were killed by a blow on the head

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and tissues immediately removed and homogenized in 10 parts of 0·25 sucrose. Supernatant fractions free of unbroken cells, nuclei and mitochondria, were prepared by centrifugation of the homogenates at 9,000 × *g* for 20 min. The microsomes were obtained by centrifugation of the supernatant for 60 min at 72,000 × *g*. Microsomes were thoroughly washed with isotonic potassium chloride and recentrifuged; they were then resuspended in potassium chloride solution, so that 1 ml of suspension contained the microsomes from 1 g of tissue.

Cyanocobalamin was incubated with the tissue preparations in different concentrations, with or without cofactors, at pH values from 5 to 8; volume was made up to 3·5 ml with 0·1M phosphate buffer.

Experiments were at 0°, 25° and 37° in a metabolic shaking incubator both in air and anaerobically, using an atmosphere of nitrogen, or *in vacuo*.

Chromatography. Samples of the incubated mixtures (0·5–0·7 ml) were put directly on thin-layer chromatoplates of silica gel (Kieselgel G Merck suspension in 95% ethanol, dried at 37°) and developed with 50% ethanol. The silica areas of hydroxo- (Rf 0·08–0·1) and cyanocobalamin (Rf 0·58–0·60) were transferred into a G4 Büchner and eluted with 50% ethanol to obtain a final concentration of about 5 μg/ml. These procedures were performed in dim light or in the dark.

Determination of cobalamins. Cyanocobalamin was determined spectrophotometrically (British Pharmacopoeia, 1963). Hydroxocobalamin binds firmly to the silica (Dony & Conter, 1956; Cima & Mantovan, 1962); accordingly, its spectrophotometric evaluation in eluates was merely qualitative. It was quantitatively determined by reconverting it to cyanocobalamin. The silica powder containing the hydroxocobalamin was suspended in 5 ml of M/15 phosphate buffer, pH 6, containing potassium cyanide (1 mg/ml) in a glass-stoppered tube; after 8–10 hr the cyanocobalamin formed was eluted as above and isolated by extraction from the buffered suspension of silica and cyanide, which was vigorously shaken three times with 50% *p*-chlorophenol (0·5 ml) in carbon tetrachloride, in a glass-stoppered centrifuge tube. The mixtures were each centrifuged and the bulked lower layers collected and shaken with n-butanol–carbon tetrachloride–water (3:1·5:0·75 the bulk volume), when the cyanocobalamin passed into the aqueous layer. The organic phase was re-extracted twice with water (0·75 × its vol.) for a quantitative recovery.

The amount of hydroxocobalamin formed could also be obtained indirectly by measuring the amount of cyanocobalamin remaining in the incubated mixture.

Both methods agreed for short incubation times. We usually used the direct method because it is independent of unrelated transformation of cyanocobalamin.

Results

In preliminary experiments, aerobically incubated homogenates (1:2 w/v) of rat liver, kidney, lung, brain, heart and spleen with cyanocobalamin

at 37° for 2 hr showed no appreciable decyanation. Anaerobically some decyanation took place with kidney and spleen (2–3%), and with liver (3–6%). Liver homogenates and anaerobic conditions were therefore used.

Since oxygen inhibits decyanation and the cyano-group can be split from cyanocobalamin by reducing agents (Kaczka, Wolf & Folkers, 1949; Fricke, Lanius, Derose, Lapidus & Frost, 1950; Brockmann, Pierce, Stokstad, Broquist & Jukes, 1950; Bernhauer, Renz & Wagner, 1962), the addition of naturally-occurring reducing compounds should enhance the formation of hydroxocobalamin. This did occur in presence of a mixture of cozymase (NADH) and flavinmononucleotide (FMN). NADH alone produced no more hydroxocobalamin than the controls; an increase did occur in the presence of FMN, though this was not so high as with the mixture of coenzymes.

Controls made without tissue homogenates showed that the coenzymes had an insignificant effect on decyanation.

The existence and properties of a "cyanocobalamin-decyanase" were elucidated by studying: (i) liver intracellular distribution; (ii) optimum conditions for activity; (iii) flavin requirement; (iv) reduced cozymase requirement; (v) influence of ions and of inhibitors; (vi) activity in kidney tissue.

(i) *Liver intracellular distribution.* Cell fractions equivalent to 0·2 g of rat liver were incubated for 1 hr at 37° with 0·7 μmole of cyanocobalamin, 10 μmole of FMN and phosphate buffer M/15, pH 7·2 under nitrogen. Almost all the enzyme activity was found in the supernatant containing the soluble fraction and the microsomes. Fractionation of this supernatant showed the decyanase was localized in the soluble fraction [cozymases are mainly localized in the soluble cell fraction (Dixon & Webb, 1964)]. Liver supernatant equivalent to 0·2 g liver (wet weight) was subsequently used.

(ii) *Optimum conditions for enzyme activity.* These were determined experimentally to be: incubation of 0·5 μmole cyanocobalamin for 90 min with 1 μmole of FMN at pH 7·2 at 37°.

(iii) *Flavin requirement.* Riboflavin, FMN and FAD were tested comparatively for their abilities to restore the decyanase activity of the soluble fraction after mild acid treatment, which is known to dissociate the flavin prosthetic group from flavoprotein (Zelitch & Ochoa, 1953).

Unexpectedly, riboflavin showed the same activity as its two coenzymes. Any of the three flavins can therefore replace the prosthetic group; furthermore, when added in excess, they markedly accelerated the extent of decyanation; they were used at a concentration of 1 μmole.

The observation that cyanocobalamin-decyanase can use riboflavin, which is usually considered only a precursor of FMN or FAD, is reminiscent of the mammalian nitroreductase system; here, activity is accelerated by the addition of an excess of any one of the flavins (Fouts & Brodie, 1957).

(iv) *Reduced cozymase requirement.* Cozymase was increased in the incubation mixtures by addition of NADH or pretreatment of the experimental animals.

Cozymase added in vitro. Some decyanation occurred when the liver supernatant was replaced by NADH, but not by NAD.

In the absence of liver supernatant, using the optimum conditions for activity for 40 min, the extent of decyanation was found to be proportional to the amount of NADH added, 1 μmole causing approximately 5·6% decyanation (Table 1). In presence of the liver supernatant, this

TABLE 1. NADH REQUIREMENT

NADH (μmole)	% Decyanation		
	Supernatant omitted (*a*)	Supernatant added	
		(*b*)	(*b*–*a*)
—	—	18·0	18·0
0·5	3·0	22·0	19·0
2·0	11·7	29·4	17·7
3·5	19·1	37·8	18·7
5·0	27·5	46·9	19·4
	Average/μmole 5·6	Average 18·5	

Anaerobic incubation for 40 min. Other conditions optimal.

became 18·5%. Decyanation to this extent might be promoted by 3·3 μmole of NADH alone. Since liver supernatant contains no more than 0·06 μmole of NADH (Ricci & Conte, 1952; Glock & McLean, 1955), its decyanating activity is about fifty times higher than expected on the basis of its NADH content. The decyanation of cyanocobalamin by NADH through reduction of the flavin therefore occurs only to a limited extent.

Effect of an increase in hepatic NADH. The *in vitro* results after NADH led us to check if an increase of liver cozymase in animals pretreated with nicotinamide increased decyanation of cyanocobalamin. Intraperitoneal administration of nicotinamide (1 g/kg) causes an average tenfold increase both of NAD and of NADH in rat liver, after 12 hr (Bonsignore & Ricci, 1948; Kaplan, Goldin, Humphreis, Ciotti & Stolzenbach, 1956; Missale & Colajacomo, 1956; Bonsignore & Ricci, 1958).

The liver supernatant from nicotinamide pretreated rats increased in decyanating ability (Table 2); this remained high for 2 hr, decreasing and

TABLE 2. INCREASE OF DECYANASE ACTIVITY IN LIVER SUPERNATANTS OF NICOTINAMIDE PRETREATED RATS

Time (hr)	Controls		Nicotinamide pretreated rats		
	Hydroxocobalamin formed (μmole)	Decyanation (%)	Hydroxocobalamin formed (μmole)	Decyanation (%)	Increase in decyanation (%)
½	0·0865	17·3	0·1205	24·1	39·3
1	0·1190	23·8	0·1650	33·0	38·6
2	0·1325	26·5	0·1790	35·8	35·0
3	0·1560	31·2	0·1865	37·3	19·5
4	0·1885	37·7	0·1940	38·8	2·9

Optimum conditions. Average values of three assays.

disappearing after 4 hr of incubation. The enzymatic activity after 30 min was similar to that obtained (see Table 1) when liver supernatant from

normal rats was added with 0·5 μmole of NADH, corresponding to a tenfold addition of the normal NADH content.

We sought the apparent relationship between the increased decyanation and the amount of NADH available. NADH cannot be measured spectrophotometrically in our system since the method does not differentiate the coenzyme from NADCN (Meyerhof, Ohlmeyer & Mohle, 1938; Colowick, Kaplan & Ciotti, 1951); we therefore followed the decrease in decyanation when the liver supernatants of the pretreated rats were stored at 0° in the air or anaerobically.

Table 3 shows that aerobic conditions cause a more rapid loss of the enzyme activity than anaerobic conditions.

TABLE 3. DECREASE OF DECYANASE ACTIVITY UPON STORAGE AT 0°

Time (days)	Aerobic	Anaerobic	Hydroxocobalamin formed (μmole)	Decyanation (%)	Decrease in decyanation (%)
–	–	+	0·1725	34·5	—
2	+	–	0·0875	17·5	49·2
2	–	+	0·1415	28·3	17·9
4	+	–	0·0420	8·4	75·6
4	–	+	0·1330	26·6	22·8
6	+	–	0·0255	5·1	85·2
6	–	+	0·1045	20·9	39·4

Incubation time 90 min; other conditions optimal. Liver supernatant of nicotinamide pretreated rats.

This suggests a definite role of the reduced form of cozymase, as a hydrogen donor; the reducing ability of NADH is likely to be maintained by pyridinenucleotide-dependent dehydrogenases and by oxidizable substrates present in the supernatant.

(v) *Influence of ions and of inhibitors.* To avoid precipitation of insoluble phosphates, 0·2M Tris-buffer was substituted for the phosphate buffer and riboflavin was used in place of FMN; previous experiments had shown that the results were unaffected.

From Table 4 it is evident that high concentrations of azide, sodium acetate, and, to a smaller extent, Fe^{++}, Fe^{+++} and pyrophosphate, inhibit the enzymatic decyanation of cyanocobalamin. Mg, Zn, Ca and Mu showed only weak inhibiting effects.

TABLE 4. INFLUENCE OF IONS AND INHIBITORS

Added compounds	μmole	Hydroxocobalamin formed (μmole)	Decyanation (%)	Inhibition (%)
—	—	0·128	25·6	—
Na-pyrophosphate	10	0·100	20·0	21·8
Na-azide	10	0·000	0·0	100·0
Na-EDTA	10	0·036	7·2	71·8
,,	50	0·031	6·2	75·7
$MgCl_2$	10	0·118	23·6	7·8
,,	100	0·120	24·0	6·2
$CaCl_2$	2·5	0·117	23·4	8·5
$FeCl_2$	10	0·064	12·8	50·0
$FeCl_3$	10	0·069	13·8	46·0
$MnCl_2$	2·5	0·122	24·4	4·6
$ZnCl_2$	2·5	0·125	25·0	2·3

Tris-buffer 0·2 M (pH 7·2). Anaerobic incubation for 120 min. Other conditions optimal.

For a better knowledge of the role of the ions involved in decyanase activity, a portion of liver supernatant was dialysed at O° for 48 hr and the activity was determined using as a reference a non-dialysed portion. A 50% reduction of the enzymatic activity was observed in the dialysed sample, which could not be restored by addition of Zn, Mg, Ca and Mn. When the dialysis was continued during 4 days, the decyanase activity disappeared completely and was not restored by the same additions. None of the ions tested was found to be essential or to act as a catalyst.

(vi) *Activity in kidney tissue.* The role of the kidney in the metabolism of cyanocobalamin is well known (Okuda & Chow, 1960; Wong, Yeh & Chow, 1960; Lee & Glass, 1961; Okuda, 1962), and we have recently shown (Cima & others, 1965; Mastrogiacomo & others, 1965) that the uptake of labelled cyanocobalamin is greater in the kidney soon after its administration than in the liver, but this is later reversed.

We found that kidney decyanating system, prepared as that of liver, is also active anaerobically and in presence of FMN or riboflavin, but it has only about 60% of liver activity.

Since the NADH content of kidney supernatant is the same as that of the liver (Glock & McLean, 1955), its low decyanating ability may be due to a smaller enzyme content or to a slower rate of activity.

It is difficult to correlate the observations *in vitro* with the higher uptake of cyanocobalamin by liver *in vivo*. But kidneys *in vivo* take up the vitamin only for a short time before undergoing a depletion (Okuda, 1962; Cima & others, 1965; Mastrogiacomo & others, 1965); furthermore cyanocobalamin is converted into coenzyme B_{12} at only about half the rate of that of the liver (Fenrych & others, 1962).

Discussion

The results suggest that cyanocobalamin is decyanated in the rat through a biochemical pathway, taking place mainly in the liver and to a lesser extent in the kidney. In both tissues there was a single or composite enzyme system converting cyanocobalamin to hydroxocobalamin; this we have called "cyanocobalamin-decyanase". It is localized in the cell soluble fraction; it is pyridine- and flavin-dependent and has a maximum activity at pH 7·2 at 37° under anaerobic conditions.

The liberated CN probably forms cyanide salts using ions from the medium and eventually an addition compound with NAD. This is known to be formed in some instances (Meyerhof & others, 1938; Colwick & others, 1951) and it may readily release the CN to hydroxocobalamin reconverting it into cyanocobalamin. Since both *in vitro* and *in vivo* decyanation were never complete, it seems that an equilibrium is reached which is probably related to the fate of the CN.

The pyridine hydrogen-donor is cozymase in its reduced form, while the enzyme includes a flavoprotein, whose prosthetic group can be replaced equally well by FAD, FMN or riboflavin. Addition of an excess of any one of these flavins accelerates the decyanation, suggesting that it not only conjugates to the enzyme but acts also as an artificial electron carrier between NADH and cyanocobalamin.

The non-specificity of the flavin requirements has been demonstrated in the biosynthesis of coenzyme B_{12} from cyanocobalamin by bacterial cell extract (Peterkofsky, Redfield & Weissbach, 1961). Also in this system, the simple role of the flavin as electron carrier was demonstrated, superseding a previous hypothesis of an alternative role as ribose donors.

Our demonstration of three of the main requirements of the system (NADH, flavin and anaerobic conditions) suggests that the conversion of cyanocobalamin to coenzyme B_{12} is a reductive process: this may involve the reduction of cobalt III to cobalt II.

Recent data report structural ambiguities concerning the cobalt atom in the coenzyme B_{12}. It appears trivalent on the basis of chemical reactions (Smith & others, 1962; Bernhauer, Müller & Müller, 1962; Müller & Müller, 1962) and of electron-spin resonance measurements (Hogenkamp, Barker & Mason, 1963), while its electrophoretic behaviour and paramagnetic character indicate that it is divalent (Johnson & Shaw, 1960; Nowichi & Pawelkiewicz, 1960; Bernhauer, Gaiser, Müller, Müller & Günter, 1961). On the other hand, vitamin $B_{12}r$, stable only under anaerobic conditions, is obtained by chemical one-electron reduction of vitamin B_{12} and contains divalent cobalt (Bernhauer, Gaiser, Müller & Wagner, 1960). Further, one-electron reduction yields vitamin $B_{12}s$ (hydridocobalamin), thought to contain monovalent cobalt. Vitamin $B_{12}s$ is also very unstable and it is the unique substrate for the chemical synthesis of coenzyme B_{12}. Decyanated cobalamins seem therefore to be the unique substrates for both chemical and enzymatic synthesis of coenzyme B_{12} (Smith & Mervyn, 1963; Johnson & others, 1963; Tackett, Collat & Abbott, 1963; Smith & others, 1964; Hill, Pratt & Williams, 1964).

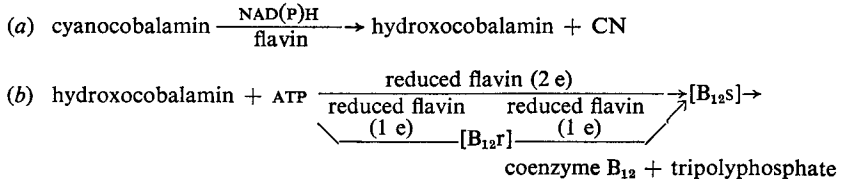
In independent experiments in which liver supernatants were incubated anaerobically in the dark at 37°, in presence of ATP for 45 hr, we did not observe the formation of coenzyme B_{12}. However Pawelkiewicz, Górna, Fenrych & Magas (1964) were able to obtain some conversion of cyanocobalamin into coenzyme B_{12} under different conditions by incubating rat liver and kidney sediments, whereas with the respective supernatants the conversion was poor (10–12%).

These observations may support the view that the synthesis of coenzyme B_{12} from cyanocobalamin in liver occurs in two stages, taking place in two individual cellular fractions. The former, in the supernatant, is the decyanase system requiring NADH and flavin; the latter, in the sediments, is a synthetase catalysing the conversion of hydroxocobalamin (via $B_{12}r$, $B_{12}s$?) and requiring the same cofactors; this one also needs ATP as a substrate and probably as a precursor of both the base and sugar moieties of the 5′-deoxyadenosyl nucleoside.

A similar two-step reaction was postulated in the enzymatic synthesis of coenzyme B_{12} by cell free extracts of some *Propionibacteria* and *Clostridia* (Pawelkiewicz, Bartosinski & Walerych, 1964). Conversely, a single-step concerted reaction was postulated by Weissbach, Redfield & & Peterkofsky (1962), because ATP itself seems necessary also for cyanide release.

Our results using liver and kidney do not agree with the last mechanism, since the release of cyanide ion from cyanocobalamin occurs independently from the addition of ATP; this, in turn, does not increase the rate of decyanation.

Our results are therefore consistent with the existence of two enzymatic processes catalysing a two-step reaction; the over-all pathway is outlined in the following scheme.



Reaction *a* has been elucidated by our studies on liver and kidney tissues; reaction *b* was postulated on the basis of more general reactions for the biosynthesis of coenzyme B_{12} in micro-organisms; a sound demonstration however is still lacking.

The hypothesis of two enzymatic processes seems to be confirmed by a recent observation of Uchino, Yagiri, Yoshino, Kondo & Wakisawa (1965) *in vivo*: carbon tetrachloride primarily injures the pathway from cyano- to hydroxocobalamin, while further transformation to coenzyme B_{12} remains almost unaffected.

The importance of decyanation as an enzymatic pathway in coenzyme B_{12} synthesis is a major conclusion of the present work. It may have a more general meaning, if it is considered in the light of its participation in the formation of methylcobalamin (methyl-B_{12}), a proposed intermediate in the biosynthesis of liver methionine (Buchanan, Elford, Loughlin, McDougall & Rosenthal, 1964; Dickerman, Redfield, Bieri & Weissbach, 1964; Weissbach & Dickerman, 1965).

References

Bernhauer, K., Gaiser, P., Müller, O. & Wagner, O. (1960). *Biochem. Z.*, **333**, 106–110.
Bernhauer, K., Gaiser, P., Müller, O., Müller, E. & Günter, F. (1961). *Ibid.*, **333**, 560–562.
Bernhauer, K., Renz, P. & Wagner, F. (1962). *Ibid.*, **335**, 443–452.
Bernhauer, K., Müller, O. & Müller, G. (1962). *Ibid.*, **336**, 102–105.
Bonsignore, A. & Ricci, C. (1948). *Boll. Soc. ital. Biol. sper.*, **24**, 170–171.
Bonsignore, A. & Ricci, C. (1958). *Scientia med. ital.*, **6**, 655–670.
Brady, R. O. & Barker, H. A. (1961). *Biochem. biophys. Res. Commun.*, **4**, 373–378.
British Pharmacopoeia (1963). Pp. 212–213.
Brockmann, J. A. Jr., Pierce, J. V., Stokstad, E. L. R., Broquist, H. P. & Jukes, T. H. (1950). *J. Am. chem. Soc.*, **72**, 1042.
Buchanan, J. M., Elford, H. L., Loughlin, R. E., McDougall, B. M. & Rosenthal, S. (1964). *Ann. N.Y. Acad. Sci.*, **112**, 756–773.
Cima, L. & Mantovan, R. (1962). *Farmaco, Ed. prat.*, **17**, 473–481.
Cima, L., Mastrogiacomo, I. & Maraschin, B. (1966). *Arch. ital. Sci. farmac.*, in the press.
Colowick, S. P., Kaplan, N. O. & Ciotti, M. M. (1951). *J. biol. Chem.*, **191**, 447–459.
Dickerman, H., Redfield, B. G., Bieri, J. G. & Weissbach, H. (1964). *Ann N.Y. Acad. Sci.*, **112**, 791–798.
Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd edn, p. 630, London: Longmans.

Dony, J. & Conter, J. (1956). *J. Pharm. Belg.*, **11**, 338–346.
Fenrych, W. J., Pawelkiewicz, J. & Magas, S. (1962). *Bull. Acad. Pol. Sci. Sèr. Sci. biol.*, **10**, 117–119.
Fouts, J. R. & Brodie, B. B. (1957). *J. Pharmac. exp. Ther.*, **119**, 197–207.
Fricke, H. H., Lanius, B., Derose, A. F., Lapidus, M. & Frost, D. V. (1950). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **9**, 173.
Glock, G. E. & McLean, P. (1955). *Biochem. J.*, **61**, 388–390.
Górna, M. (1963). *Bull. Acad. Sci. pol. Sèr. Sci. biol.*, **11**, 205–208.
Hill, H. A. O., Pratt, J. M. & Williams, R. J. P. (1964). *Chemy Ind.*, 197.
Hogenkamp, H. P. C. (1964). *Ann. N.Y. Acad. Sci.*, **112**, 552–564.
Hogenkamp, H. P. C., Barker, H. A. & Mason, H. S. (1963). *Archs Biochem. Biophys.*, **100**, 353–359.
Johnson, A. W. & Shaw, N. (1960). *Proc. chem. Soc.*, 420.
Johnson, A. W., Mervyn, L., Shaw, N. & Smith, E. L. (1963). *J. chem. Soc.*, 4146–4156.
Kaczka, E. A., Wolf, D. E. & Folkers, K. (1949). *J. Am. chem. Soc.*, **71**, 1514–1515.
Kaplan, N. O., Goldin, A., Humphreis, S. R., Ciotti, M. M. & Stolzenbach, F. E. (1956). *J. biol. Chem.*, **219**, 287–298.
Lee, D. H. & Glass, G. B. J. (1961). *Proc. Soc. exp. Biol. Med.*, **107**, 293–296.
Mastrogiacomo, I., Cima, L. & Maraschin, B. (1965). *Arch. ital. Sci. farmac.*, in the press.
Meyerhof, O., Ohlmeyer, P. & Möhle, W. (1938). *Biochem. Z.*, **297**, 113–133.
Missale, G. & Colajacomo, A. (1956). *Boll. Soc. ital. Biol. sper.*, **32**, 769–772.
Müller, P. & Müller, G. (1962). *Biochem. Z.*, **336**, 299–313.
Nowichi, L. & Pawelkiewicz, J. (1960). *Bull. Acad. pol. Sci. Sèr. Sci. biol.*, **8**, 433–434.
Okuda, K. & Chow, B. F. (1960). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **19**, 417.
Okuda, K. (1962). *J. Nutr.*, **77**, 131–136.
Pawelkiewicz, J., Bartosiński, B. & Walerych, W. (1964). *Ann. N.Y. Acad. Sci.*, **112**, 638–640.
Pawelkiewicz, J., Górna, M., Fenrych, W. & Magas, S. (1964). *Ibid.*, **112**, 641–643.
Peterkofsky, A., Redfield, B. & Weissbach, H. (1961). *Biochem. biophys. Res. Commun.*, **5**, 213–216.
Peterkofsky, A. & Weissbach, H. (1964). *Ann. N.Y. Acad. Sci.*, **112**, 622–637.
Ricci, C. & Conte, G. (1952). *Boll. Soc. ital. Biol. sper.*, **28**, 297–298.
Smith, E. L., Mervyn, L., Johnson, A. W. & Shaw, N. (1962). *Nature, Lond.*, **194**, 1175.
Smith, E. L. & Mervyn, L. (1963). *Biochem. J.*, **86**, 2P–3P.
Smith, E. L., Mervyn, L., Muggleton, P. W., Johnson, A. W. & Shaw, N. (1964). *Ann. N.Y. Acad. Sci.*, **112**, 565–574.
Tackett, S. L., Collat, J. W. & Abbott, J. C. (1963). *Biochemistry*, **2**, 919–923.
Toohey, J. I. & Barker, H. A. (1961). *J. biol. Chem.*, **236**, 560–563.
Uchino, H., Yagiri, Y., Yoshino, T., Kondo, M. & Wakisawa, G. (1965). *Nature, Lond.*, **205**, 176–177.
Weissbach, H., Redfield, B. & Peterkofsky, A. (1962). *J. biol. Chem.*, **237**, 3217–3222.
Weissbach, H. & Dickerman, H. (1965). *Physiol. Rev.*, **45**, 80–97.
Wong, V., Yeh, S. & Chow, B. (1960). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **19**, 415.
Zelitch, I. & Ochoa, S. (1953). *J. biol. Chem.*, **201**, 707–718.