CHROM. 3484

THE DETECTION AND IDENTIFICATION OF INTERMEDIATES OF THE PENTOSE PHOSPHATE CYCLE AND RELATED COMPOUNDS

T. WOOD
Department of Biochemistry, McGill University, Montreal 2, Que. (Canada)
(Received February 26th, 1968)

SUMMARY

Procedures are described for precipitating with barium, the intermediates of the pentose phosphate cycle and some related compounds. Following the removal of the barium the phosphates can be chromatographed on paper as their sodium salts, and detected with specific colour reagents. The identity of the sugar moiety can be confirmed by phosphatase hydrolysis. R_F values of these compounds and the parent sugars in 4 solvents are given. A new colour reaction for erythrose-4-phosphate is reported.

In 1960 I published a procedure[1] for the analysis, by paper chromatography, of the acid-soluble phosphorous compounds in animal tissues which has been applied and found useful in a variety of situations[2–5]. Recently, these methods have been modified and extended to permit separation and identification of the intermediates of the pentose phosphate cycle. Since there is little information in the literature on ways of handling and chromatographing intermediates of the cycle, the techniques finally adopted are described below.

MATERIALS AND EQUIPMENT

Ascending chromatography was carried out on 10 in. square sheets of Whatman paper in a Shandon Smith Universal Chromotank*. The papers were mounted on polythene frames, able to carry a maximum of 5 papers, and all accessories were of polythene. Some chromatograms were run in glass jars with metal screw-tops which had a thin sheet of polythene interposed between the top of the jar and the metal lid. The papers were rolled into a cylinder, the edges held together with a polythene clip, and the cylinder stood in the solvent in the bottom of the jar.

Desalting of phosphatase hydrolysates was done in an ion-exchange membrane electrodialyzer*[6].

The sodium salt of ribose-5-phosphate, the lithium salt of hydroxypyruvic acid, the potassium salt of glucose-6-phosphate, D-erythrose and sedoheptulosan, were

* Obtained from Shandon through Consolidated Laboratories (Canada) Ltd., 66 Milvan Drive, Weston, Ont.

all obtained from the Nutritional Biochemical Corporation. The magnesium salt of phosphoribosyl pyrophosphate and ribulose diphosphate dibarium salt were from Sigma. Erythrose-4-phosphate dimethylacetal and ribulose-O-nitrophenylhydrazone (used as a colorimetric standard in the cysteine–carbazole reaction) were supplied by Calbiochem. The tri-monocyclohexylammonium salt of 6-phosphogluconic acid was a product of Boehringer Mannheim Corporation. The sources of enzymes are described in the text.

REFERENCE COMPOUNDS

Since a number of the compounds required are not available commercially, they were prepared by previously published methods, slightly modified. Where possible, commercial enzyme preparations were used.

Ribulose-5-phosphate

The tri-monocyclohexylammonium salt of 6-phosphogluconic acid (100 μmoles), 150 μmoles of potassium pyruvate, 6 μmoles of NADP, 180 units of rabbit muscle lactic dehydrogenase (Boehringer crystalline enzyme, specific activity 360 international units/mg) and 4.8 units of yeast 6-phosphogluconic dehydrogenase (Boehringer enzyme specific activity 12 international units/mg) were incubated at 30° and pH 7.4 in a total volume of 6.0 ml. Samples were withdrawn at intervals and the ribulose-5-phosphate estimated by reaction with cysteine and carbazole[7]. When the colour obtained had reached a maximum (after approx. 90 min) the mixture was cooled in ice, the pH adjusted to 6.6 with acetic acid and 25 % w/v barium acetate added until there was no further precipitate. The precipitate was centrifuged down, washed twice with 1.0 ml of water and the combined washings and supernatant adjusted to pH 6.6 and transferred to a 50 ml stoppered cylinder. To the solution was added 5 volumes of ice-cold absolute alcohol and a few drops more of 25 % barium acetate. After standing overnight at 0° the precipitated barium salt was removed by centrifuging and converted to the sodium salt as described later.

Ribulose-5-phosphate was also prepared by incubating sodium ribose-5-phosphate with spinach phosphoriboisomerase (Sigma Type I, specific activity 55 international units/mg) at pH 7.4 and 37°. After deproteinisation with 3 % w/v perchloric acid the solution was neutralized with potassium hydroxide to pH 6.5 and lyophilized. The commercial enzyme was almost free of xylulose-5-phosphate epimerase and gave 25 % conversion to ribulose-5-phosphate at equilibrium.

Xylulose-5-phosphate, sedoheptulose-7-phosphate

A sample of ribulose-5-phosphate prepared from 6-phosphogluconate was reacted with xylulose-5-phosphate epimerase prepared from rabbit muscle by the procedure of SRERE *et al.*[8]. The epimerase was assayed for phosphoriboisomerase activity[9] and found to be free of this enzyme. Since xylulose-5-phosphate gives only 1/3 of the colour obtained with an equivalent amount of ribulose-5-phosphate in the cysteine–carbazole reaction[10], its formation was followed from the steady fall in colour given by aliquots of the reaction mixture. When a position of equilibrium had been reached the solution was deproteinized, neutralized, and lyophilized. A mixture of ribulose-5-phosphate and xylulose-5-phosphate was obtained.

A mixture of xylulose-5-phosphate and sedoheptulose-7-phosphate was obtained by incubation of lithium hydroxypyruvate and fructose-1,6-diphosphate with aldolase and transketolase as described by SRERE *et al.*[8]. The incubation mixtures contained 12 m*M* N-ethylmaleimide to partially inhibit phosphoriboisomerase and xylulose-5-phosphate epimerase present in the transketolase preparation[11]. The progress of the reaction was followed by withdrawing aliquots and reacting them with ferric chloride–orcinol[12]. Measurement of the increase in optical density at 670 mμ and at 580 mμ gave an approximate measure of pentose phosphate and sedoheptulose phosphate formation respectively. At the end of the incubation period, the mixture was deproteinized with perchloric acid, neutralized to pH 6.5 with potassium hydroxide and lyophilized.

Xylulose and ribulose

D-Xylulose and D-ribulose were prepared from D-xylose and D-ribose respectively by refluxing the sugars in dry pyridine as described by SCHMIDT AND TREIBER[13].

Sedoheptulose

A mixture of sedoheptulose and sedoheptulosan was prepared by standing sedoheptulosan in 3 *N* HCl for 12 h[14].

Erythrose-4-phosphate

This was regenerated from the dimethylacetal by the method described by the suppliers.

EXPERIMENTAL METHODS

In view of the fact that two important members of the pentose phosphate cycle, ribulose-5-phosphate and xylulose-5-phosphate are very alkali-labile[15–17], the procedures described earlier for concentrating and chromatographing mixtures of phosphates were modified to allow the detection of these compounds.

Extraction and precipitation of barium salts

The barium salts of the ketopentose phosphates are very soluble and for their successful precipitation the initial extract containing the phosphorylated compounds must not be too dilute. The acid extracting agent (3 % w/v perchloric acid) was neutralized carefully with potassium hydroxide or potassium bicarbonate and the pH was never allowed to rise above 6.0–6.5. The extract was kept cold in ice and 25 % w/v barium acetate was added until there was no further precipitate, then a drop of bromocresol purple was added followed by sufficient dilute potassium hydroxide to produce a pale purple colour (pH 6.2–6.4). After spinning down the precipitate, a further drop of barium acetate was added and the process continued adding alternately dilute alkali and barium acetate until there was no further precipitate. During this procedure the pH was not allowed to rise above 6.5. Finally, the mixture was transferred to a cold stoppered cylinder and 5 volumes of ice-cold absolute alcohol were added together with more indicator and sufficient alkali to just bring the colour back to pale purple. The cylinder was stoppered and stood in the refrigerator with occasional shaking for 30 min. At the end of this time, the precipitate was allowed to settle and

the supernatant was tested with barium acetate to see whether precipitation was complete and an excess of barium acetate was present. The mixture was allowed to stand with occasional shaking for 8 h. If complete precipitation of the barium salts appeared difficult to achieve, 10 % v/v ether was added.

The barium salts were centrifuged down and redissolved or resuspended in a small volume of water. The phosphorylated compounds were converted to their sodium salts by adding Zeo-Karb 226* in the sodium form followed by vigorous stirring. Successful exchange was detected by the almost complete absence of insoluble material after centrifuging and the lack of a precipitate when a drop of the supernatant was tested with 2 *N* H_2SO_4. Sometimes, it was found necessary to separate the supernatant containing suspended insoluble material from the bulk of the resin and treat it with fresh resin before clear solutions could be obtained. The addition of a few drops of 2 *N* acetic acid during the resin treatment also facilitated the exchange, on occasion.

When all the barium had been exchanged for sodium, the supernatant was removed from the resin which was then washed. The supernatant and washings were then combined, clarified by centrifuging, the pH adjusted to 6.0–6.5 and the solution freeze-dried. The freeze-dried residue was redissolved in a small volume of water, the pH again checked and the concentrated solution of phosphates stored at —20°. Since it was found that the pH of the solution sometimes changed on storage, the pH of the solutions was checked periodically and readjusted to the safe range of pH 6.0–6.5 when necessary.

Hydrolysis with acid phosphatase

The procedure of DICKENS AND WILLIAMSON[16] was followed except that larger amounts of material were hydrolysed. Up to 10 μmoles of pentose phosphate was incubated with 0.1 ml of acid phosphatase (Boehringer potato acid phosphatase, Type II, 1 mg/ml) at 37° in a total volume of 2.0 ml 0.05 *M* acetate buffer, pH 5.0, containing 5 m*M* magnesium. Aliquots of 0.01 ml were withdrawn at intervals and inorganic phosphate or ketopentose determined. The hydrolysis was usually complete after 4–6 h.

Following deproteinization with perchloric acid and neutralization to pH 7.0, the protein and potassium perchlorate were removed and the solution was desalted in a WOOD ion-exchange membrane electrodialyzer[6]. After this treatment the solution (pH 6.5–7.0) was freeze-dried and redissolved in a small volume prior to chromatography.

Paper chromatography

Whatman No. 541 paper was used for the separation of the phosphates and Whatman No. 1 for chromatography of the free sugars. Solutions were applied to the paper with a platinum wire loop and the spot dried by a blast of cold air.

Solvents for sugar phosphates

(1) *n*-Butanol–*n*-propanol–acetone–80 % (w/v) formic acid–30 % (w/v) trichloracetic acid (40:20:25:25:15 by vol.). This is solvent GW3 described previously and is run twice in the same direction[1,18].

(2) Methanol–90 % formic acid–water (80:15:5 by vol.)[19,20].

* Rexyn 102 may also be used.

(3) Propyl acetate–90 % (w/v) formic acid–water (55:25:15 by vol.)[21]. This solvent was also run twice.

To the above solvents 0.05 g of the tetrasodium salt of EDTA was added for each 100 ml of solvent.

Solvent for free sugars

(4) 80 % (w/v) Phenol[22].

(5) Isopropanol–*n*-butanol–water (70:10:20 by vol.)[22].

DETECTION REAGENTS

Acid molybdate

Ammonium molybdate (1 g) was finely ground and dissolved in 8.0 ml of water, 3.0 ml conc. HCl and 3.0 ml 70 % perchloric acid were added. One quarter of this mixture (3.5 ml) was added to 22 ml of acetone. Papers were dipped through this solution and, when the acetone had evaporated, were irradiated in U.V. light in a humid atmosphere. Phosphorylated compounds appeared as blue spots.[1]

3,4-Dinitrobenzoic acid

The properties of this reagent have been described in detail[24]. It gave a violet colour with ribulose-5-phosphate, xylulose-5-phosphate, erythrose-4-phosphate and with dihydroxyacetone phosphate. It did not react with ribose-5-phosphate.

Orcinol

The reagent[25] gave a characteristic blue green colour with sedoheptulose-7-phosphate, sedoheptulose, and sedoheptulosan. It also gave a grey-green spot with ribulose and a purple black colour with xylulose. After a further treatment with aniline phosphate[23] at room temperature the ribulose spot turned pink with an intense orange fluorescence under U.V. light[26,27]; in contrast, the grey-purple xylulose spot did not change colour or fluoresce.

Phloroglucinol

Ribose and ribose-5-phosphate gave rose colored spots with this reagent[28] while the ketopentose phosphates did not react. Sedoheptulose-7-phosphate gave an olive-green colour.

Urea/HCl

A modified form of this reagent[29] was prepared by mixing 2.5 g urea, 10 ml 6 *N* HCl and 50 ml of ethanol. The chromatograms were dipped, dried and heated 2 min at 95°. A purple colour was produced with ribulose-5-phosphate when large amounts were present but it was most useful for detecting erythrose-4-phosphate which gave a characteristic pale rose-colour and amounts as small as 5 μg could be detected[30]. Free erythrose gave only a pale brown colour.

Triphenyltetrazolium

All sugars and sugar phosphates with a reducing group react with triphenyltetrazolium[1]. Ribose-5-phosphate, ribulose-5-phosphate and xylulose-5-phosphate reacted in the cold without exposure to steam.

RESULTS AND DISCUSSION

The phosphorylated sugars listed in Table I were stable in the solvents used and could be preserved on storage for many weeks in the form of their sodium salts provided that the pH of the solutions was maintained below 7.0.

As ribulose-5-phosphate and xylulose-5-phosphate are easily broken down under alkaline conditions only acid or neutral solvents may be used for their chromatography. The ammonium isobutyrate solvent described previously[1] could not be used since ribulose-5-phosphate was decomposed by it, probably by reaction with the ammonia of ammonium isobutyrate deposited on the paper when the chromatograms were dried. In the usual procedure for the precipitation of organic phosphates as their barium salts, alkali is added until there is a pink reaction with phenolphthalein (pH 8.0–8.5). This procedure destroyed ribulose-5-phosphate, so bromocresol purple was substituted for phenolphthalein and successful precipitation of the ketopentose phosphates was achieved.

TABLE I

R_F VALUES OF SUGAR PHOSPHATES

Sugar phosphate	R_{PO_4} *GW3*[2]	R_F *Methanol–formic acid*
P_i	1.00	0.72
6-PG	0.49	0.53
G-6-P	0.41	0.40
R-5-P	0.55	0.57
Ru-5-P	0.55	0.61
Xu-5-P	0.55	0.52
Er-4-P	0.57	0.64
DHAP	0.72	0.65
GAP	0.74	0.60
Ru-1,5-PP	0.43	0.54
PRPP	0.40	0.36 streaks
S-7-P	0.38	0.43

* Abbreviations used above and elsewhere in this paper: P_i = inorganic phosphate; 6-PG = 6-phosphogluconic acid; G-6-P = glucose-6-phosphate; R-5-P = ribose-5-phosphate; Ru-5-P = ribulose-5-phosphate; Xu-5-P = xylulose-5-phosphate; Er-4-P = erythrose-4-phosphate; DHAP = dihydroxyacetone phosphate; GAP = glyceraldehyde phosphate; Ru-1,5-PP = ribulose-1,5-diphosphate, PRPP = phosphoribulose pyrophosphate; S-7-P = sedoheptulose-7-phosphate.

The only reported separation on paper of Ru-5-P and Xu-5-P that could be traced in the literature was published recently by LIONETTI AND FORTIER[31] using the solvent of RUNECKLES AND KROTKOV[32]. In the hands of the author, this solvent gave long streaks but, fortunately, an adequate separation of the two isomeric ketopentose phosphates could be obtained with methanol–formic acid and with propyl acetate–formic acid. However, certain precautions had to be taken, otherwise the two compounds ran together as a single spot: the solvent had to be allowed to esterify for 2 to 3 days before use, and it had to be run at *right angles* to the machine direction of the paper.

The R_F values of ribulose-5-phosphate and xylulose-5-phosphate in methanol–

formic acid were affected by the presence of salts and other substances in the mixture being chromatographed. This sometimes resulted in these compounds travelling either more slowly, or faster, than usual, and a failure to resolve a mixture of the two ketopentose phosphates into separate spots. Thus, when a pure sample of ribulose-5-phosphate was added to an impure sample of xylulose-5-phosphate, a portion of the added ribulose-5-phosphate was retarded and increased the intensity of the lower spot. The presence of ribulose-5-phosphate and xylulose-5-phosphate together in the spot could be detected by a second run at right angles to the first in the same solvent. Instead of a single spot due to xylulose-5-phosphate lying along the diagonal of the paper, two spots were obtained, one lying on the diagonal (xylulose-5-phosphate) and the other (ribulose-5-phosphate) further to one side (see Fig. 1).

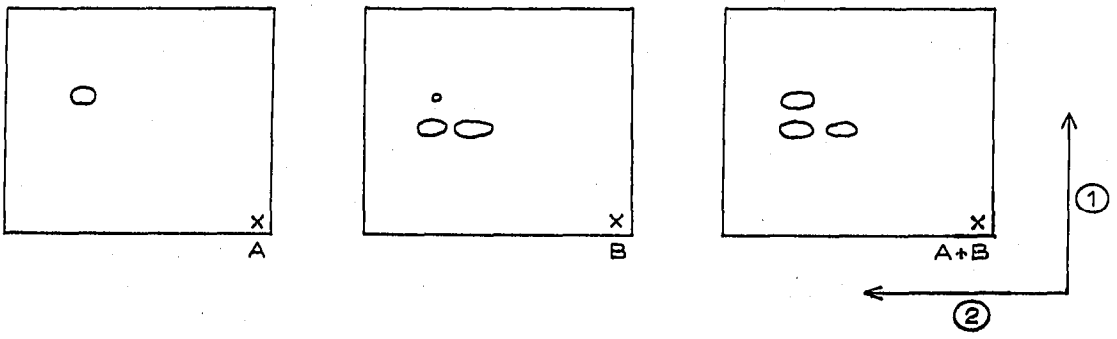

Fig. 1. Two-dimensional chromatography of the ketopentose phosphates using methanol–formic acid–water in both dimensions 1 and 2. A = ribulose-5-phosphate prepared from 6-phosphogluconate. B = xylulose-5-phosphate/ribulose-5-phosphate mixture prepared by the action of phosphoketopentoepimerase on the above sample of ribulose-5-phosphate.

A propyl acetate–formic acid solvent run twice, was also used for the separation of the two isomeric ketopentose phosphates. Although the R_F values were not great (Ru-5-P = 0.27, Xu-5-P = 0.22), in general, adequate separations were obtained. The presence of other compounds in the sample had no effect on these R_F values, probably because of the slower rate of migration in this solvent. Furthermore, two-dimensional chromatograms of mixtures of xylulose-5-phosphate and ribulose-5-phosphate using this solvent in both dimensions gave only two spots situated on the diagonal of the paper, and addition of ribulose-5-phosphate to the sample intensified only the upper spot.

In view of the difficulties encountered in separating these two substances and the erratic behaviour described above, it is recommended that they be identified from their positions on the chromatogram only after comparison and co-chromatography with known markers and that, whenever possible, confirmation should be obtained from the phosphatase hydrolysis procedure mentioned below.

An accepted method for identifying the pentose phosphates is hydrolysis with phosphatase followed by paper chromatography in phenol and production of the characteristic colour reactions of the free sugars. Following hydrolysis with phosphatase, salts and inorganic phosphate were successfully removed by electrodialysis with almost no loss of sugar. Both 80% phenol and isopropanol–butanol gave good separations of ribulose and xylulose and of the other sugars listed in Table II. However, chromatograms had to be hung for several days to remove traces of phenol

which otherwise diminished the sensitivity of the triphenyltetrazolium and 3,4-dinitrobenzoic acid reagents. The isopropanol–butanol solvent did not have this disadvantage and possessed also the useful property of reversing the relative positions of xylulose and ribulose.

TABLE II

R_F VALUES OF SUGARS

Sugar	R_F	
	80 % Phenol	*Isopropanol–butanol*
Xylulose	0.63	0.50
Ribulose	0.67	0.45
Ribose	0.62	0.43
Xylose	0.48	0.37
Sedoheptulose	0.41	0.28
Sedoheptulosan	0.73	0.28
Erythrose	0.67	0.52

Certain colour reagents were found very useful in that they gave, on paper, specific and characteristic reactions with a number of the phosphates of interest, although unfortunately, no reagent could be found that would give different colours with the two isomeric ketopentose-phosphates. Phloroglucinol gave a purple colour with ribose-5-phosphate and did not react with the ketopentose phosphates, while the latter, together with erythrose-4-phosphate, yielded a violet colour with alkaline 3,4-dinitrobenzoic acid but ribose-5-phosphate gave no colour. Erythrose-4-phosphate gave a pale rose spot with urea–hydrochloric acid and sedoheptulose-7-phosphate gave the well-known characteristic blue-green colour with the orcinol reagent. The 3,4-dinitrobenzoic acid reagent was particularly useful in demonstrating the activity of the pentose phosphate cycle in a given tissue since when chromatograms of the phosphates extracted from a tissue such as spleen or liver were treated with the reagent, violet spots due to ketopentose phosphates appeared, while chromatograms of similar extracts from tissues such as muscle that have little or no pentose phosphate cycle activity gave no spots with this reagent.

The procedures outlined above have been applied successfully to the following: (1) the products of the action of xylulose-5-phosphate epimerase on ribulose-5-phosphate; a typical chromatogram showing the phosphates present after incubation of ribose-5-phosphate with an extract of rat muscle is shown in Fig. 2. (2) the product of the action of partially purified phosphopentoisomerase and xylulose-5-phosphate epimerase on ribose-5-phosphate; (3) the phosphates formed from hydroxypyruvate and fructose diphosphate by the action of partially purified spinach transketolase and purified aldolase and triose phosphate isomerase; (4) mixtures of the phosphates arising from the action of extracts of acetone-dried powders of Ehrlich ascites cells, and rat muscle, on ribose-5-phosphate, as described by DICKENS AND WILLIAMSON[16]; (5) extracts of Ehrlich ascites cells; (6) extracts of red blood cells.

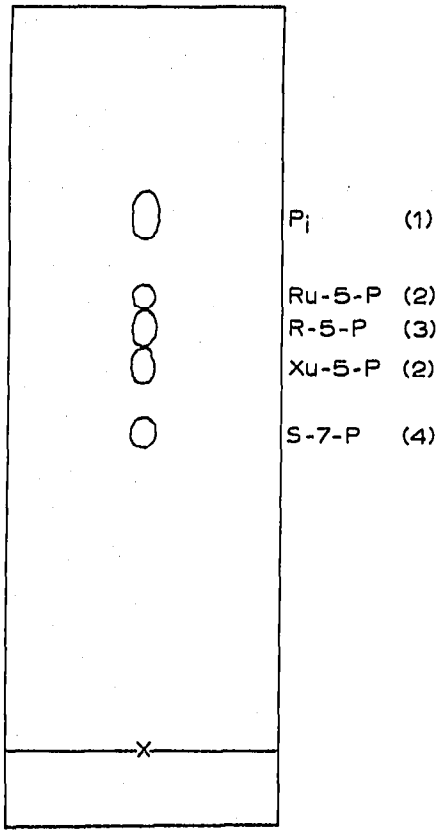

Fig. 2. One dimensional chromatogram in the methanol–formic acid solvent of the phosphates resulting from the incubation of a rat muscle extract with ribose-5-phosphate. (1) spot giving a yellow colour with the phosphate reagent before U.V. irradiation; (2) spots giving a violet colour with the 3,4-dinitrobenzoic acid reagent; (3) spot giving a purple colour with phloroglucinol; (4) spot giving a green colour with phloroglucinol and a green colour with orcinol.

ACKNOWLEDGEMENTS

I wish to thank Mr. E. L. TAN who provided the xylulose-5-phosphate epimerase and Miss DAWNE E. ABRAHAMS for preparing spinach transketolase. This work was supported by grant MA 2205 from the Medical Research Council of Canada.

REFERENCES

1 T. WOOD, *J. Chromatog.*, 6 (1961) 142.
2 T. WOOD, *J. Chromatog.*, 8 (1962) 475.
3 Y. QURESHI AND T. WOOD, *Biochim. Biophys. Acta*, 60 (1962) 190.
4 T. WOOD, *J. Sci. Food Agr.*, 12 (1961) 61.
5 O. GARCIA-AROCHA AND T. WOOD, *Proc. Can. Fed. Biol. Soc.*, 10 (1967) 105.
6 T. WOOD, *Biochem. J.*, 62 (1956) 611.
7 Z. DISCHE AND E. BORENFREUND, *J. Biol. Chem.*, 192 (1951) 583.
8 P. SRERE, J. R. COOPER, M. TABACHNIK AND E. RACKER, *Arch. Biochem. Biophys.*, 74 (1958) 295.
9 B. AXELROD AND R. JANG, *J. Biol. Chem.*, 209 (1954) 847.
10 G. ASHWELL AND J. HICKMAN, *J. Biol. Chem.*, 226 (1957) 65.
11 M. TABACHNIK, P. A. SRERE, J. COOPER AND E. RACKER, *Arch. Biochem. Biophys.*, 74 (1958) 315.

12 B. L. HORECKER, in S. P. COLOWICK AND N. O. KAPLAN (Editors), *Methods of Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 105.
13 O. T. SCHMIDT AND R. TREIBER, *Chem. Ber.*, 66 (1933) 1765.
14 L. UJESKI AND E. R. WAYGOOD, *Can. J. Chem.*, 32 (1954) 14.
15 S. COHEN, *J. Biol. Chem.*, 201 (1953) 71.
16 F. DICKENS AND D. H. WILLIAMSON, *Biochem. J.*, 64 (1956) 567.
17 J. HURWITZ AND B. L. HORECKER, *J. Biol. Chem.*, 223 (1956) 993.
18 E. GERLACH, E. WEBER AND H. J. DÖRING, *Arch. Exptl. Pathol. Pharmakol.*, 226 (1955) 9.
19 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
20 R. S. BANDURSKI AND B. AXELROD, *J. Biol. Chem.*, 193 (1951) 405.
21 R. L. BIELESKI, *Anal. Biochem.*, 12 (1965) 230.
22 I. SMITH, in I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Vol. 1, Heinemann and Interscience, London and New York, 1960, p. 248.
23 I. SMITH, in I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Vol. 1, Heinemann and Interscience, London and New York, 1960, p. 251.
24 T. WOOD AND DAWNE E. ABRAHAMS, *Anal. Biochem.*, 22 (1968) 117.
25 R. KLEVSTRAND AND A. NORDAL, *Acta Chem. Scand.*, 4 (1950) 1320.
26 S. S. COHEN AND D. B. M. SCOTT, *Science*, 111 (1950) 543.
27 R. M. HOCHSTER, *Can. J. Microbiol.*, 1 (1955) 346.
28 E. BORENFREUND AND Z. DISCHE, *Arch. Biochem. Biophys.*, 67 (1957) 239.
29 R. DEDONDER, *Compt. Rend.*, 230 (1950) 997.
30 T. WOOD, unpublished observations.
31 F. J. LIONETTI AND N. L. FORTIER, *Arch. Biochem. Biophys.*, 103 (1963) 15.
32 V. C. RUNECKLES AND G. KROTKOV, *Arch. Biochem. Biophys.*, 70 (1957) 442.