was relatively small. With increase in time of exchange, the specific radioactivity of the trichloroacetic acid-soluble fraction will no longer remain proportional to the true specific radioactivity of free cyst(e)ine, and therefore, it cannot be used for calculation of turnover rates.

SUMMARY

1. Measurements were made of the labelling of fractions containing (a) proteolipid, (b) other protein and (c) free amino acids of the rat brain after administering L-[³⁵S]methionine *in vivo*.

2. The rate of incorporation of ³⁵S into the proteolipids was much lower than the rate of incorporation into the other proteins. The radioactivity of the lipid fraction was present mainly in the amino acids of the protein component of the proteolipids; the radioactivity in the sulphatide fraction was relatively small.

3. A comparison of the apparent turnover rates of [³⁵S]methionine and [³⁵S]cystine into brain proteins at short intervals after injection of [³⁵S]methionine suggested that demethylation of [³⁵S]methionine was probably not limiting the rate of incorporation of [³⁵S]cystine.

4. The apparent turnover rate of [³⁵S]methionine into brain proteins increased with time of exchange of the injected [³⁵S]methionine. The significance of this observation has been discussed.

I am indebted to Dr Jordi Folch for his interest and encouragement during the course of this work. I also wish to thank him and Dr Derek Richter for their valuable suggestions in the preparation of the manuscript. Thanks are also due to Miss June Bellotti for her technical assistance. This work was supported by a grant from the Ford Foundation.

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Biochem. J. (1961) **80**, 284

The Chromatography of Normal Serum Proteins

By M. P. TOMBS, K. B. COOKE, D. BURSTON AND N. F. MACLAGAN

Department of Chemical Pathology, Westminster Medical School, Horseferry Road, London, S.W. 1

(Received 18 October 1960)

Substituted celluloses are now in widespread use for the chromatography of protein, the most common being diethylaminoethylcellulose and carboxymethylcellulose (Sober & Peterson, 1956), and numerous applications to serum proteins have been described (for a review see Sober & Peterson,

1958). However, chromatography of serum proteins on diethylaminoethylcellulose so far described has failed to resolve them into components homogeneous even as judged by the relatively simple criterion of paper electrophoresis (Sober & Peterson, 1956; Fahey, McCoy & Goulian, 1958).

Similar results have been obtained with carboxymethylcellulose (Cooke, Tombs, Souter, Weston & MacLagan, 1959).

The detailed electrophoretic analysis of chromatographic fractions (Fahey *et al.* 1958) has shown that, whereas both the α - and β -globulins are resolved into a number of subfractions on diethylaminoethylcellulose columns, the α -globulin subfractions are not separated from those of the β -globulins. Cooke *et al.* (1959) showed the utility of ammonium sulphate fractionation followed by diethylaminoethylcellulose chromatography for separation of the more soluble proteins, but indicated that the less soluble α - and β -globulins were poorly resolved. Alternating chromatography on carboxymethyl- and diethylaminoethyl-cellulose has found some application, but suffers from practical difficulties which restrict its use for serum protein. It appeared, therefore, that a preliminary electrophoretic separation combined with column chromatography might provide the best approach to the fractionation of the serum proteins, and we present here the results of the chromatographic analysis of protein fractions obtained by starch-grain electrophoresis.

Although 280 $m\mu$ is the wavelength usually chosen for the estimation of protein in chromatographic eluates, the use of a wavelength of 210 $m\mu$ permits the detection of much smaller quantities of protein (Tombs, Souter & MacLagan, 1959). By scanning the eluate at this wavelength the chromatographic analysis of the fractions obtained by the paper electrophoresis of 0.2 ml. of serum was feasible.

MATERIALS AND METHODS

Serum. Blood was drawn in 50 ml. batches from normal subjects by venipuncture and immediately clotted with thrombin. This proved to be the best way of avoiding haemolysis.

Analytical electrophoresis. Paper electrophoresis was performed in borate buffers (13.2 g. of sodium tetraborate and 6.99 g. of boric acid/l.; I 0.075; pH 8.3) in a vertical tank. It was important to avoid veronal buffers where a paper electrophoresis run was to be followed by chromatography as veronal buffers absorb strongly at 210 $m\mu$. Immunoelectrophoresis was carried out in agar by a slight modification of the method of Scheidegger (1955), applying approximately 5 μ l. of solution to each well. A horse anti-human serum (Institut Pasteur, Paris) was used. Lipoproteins were detected by staining with oil-red O, and haemoglobin or haematin-combining proteins by staining with benzidine. The same anti-serum and agar as were used for immunoelectrophoresis were used for Ouchterlony plate diffusions. The results shown in the figures were obtained by tracing a projection of the stained slides.

Preparative electrophoresis. Of several methods tried, the most successful, and that finally adopted, was electrophoresis on starch grains (Kunkel, 1954). A slab (40 cm. $\times$

14 cm. $\times$ 2 cm.) of washed starch (British Drug Houses Ltd.; laboratory-reagent grade), twice washed and finally suspended in borate buffer (I 0.075; pH 8.3) was held between cooling tanks through which aqueous ethylene glycol coolant at -2° was circulated. With a potential gradient of 5 v/cm. along the slab and a current of 150 ma the temperature never rose above 10° and the electrophoretic run was completed in 6–7 hr. Each slab (holding 10–15 ml. of serum) was sectioned after electrophoresis, by using the coloured zones as a guide, into albumin (yellow), α_2 - (blue due to caeruloplasmin), β - (pink due to siderophilin) and γ -globulins (these lie behind the sample slot).

Protein was eluted from each section, usually with the chromatography equilibration buffer, by packing the starch into a column fitted with a sintered-glass disk and applying gentle suction. The fractions were then checked for homogeneity by paper electrophoresis. The α_2 -globulin always contained a small amount of albumin and the albumin fraction some α_1 - and α_2 -globulins. Haemolysed serum failed to give a satisfactory separation.

Column chromatography. Diethylaminoethylcellulose (DEAE-cellulose) and carboxymethylcellulose (CM-cellulose) were prepared according to Sober & Peterson (1956). Solka Floc SW 40B, sieved through 100 mesh, was used for DEAE- and Solka Floc BW 200 for CM-cellulose. Solka Floes are manufactured by the Brown Corp., Berlin, N.H., U.S.A. BW 200 was obtained through V. A. Howe Ltd., 46 Pembridge Road, London, W. 11, and SW 40B was a gift from Imperial Chemical Industries Ltd., Nobel Division, Manchester.

Columns were packed under air pressure, though it was important not to apply pressure until the column material had settled, otherwise sintered disks became clogged. The pressure required for optimum flow rates depended upon the batch of column material used. Usually 5 lb./in.² gave adequate flow rates. Increasing the pressure above about 7 lb./in.² decreased flow rates. Before packing, the column material was almost equilibrated with the appropriate buffer by dialysis. Final equilibration was then carried out by running buffer through from the reservoir *A* (Fig. 1) until the pH of the effluent was the same as that of the inflow. The general arrangement of the column is shown in Fig. 1. Protein solutions, previously equilibrated by dialysis with the same buffer, were then carefully applied to the top of the column and allowed to run in under gravity, or driven in under pressure if the volume was exceptionally large (over 50 ml.). The volume of applied solution had no effect on subsequent results. Elution was then carried out, either stepwise with reservoir *A* or by gradient. The gradient arrangement shown used two flasks *B* and *C*, connected by a syphon so that the levels always remained constant. The cone-sphere system gives concave gradients which are particularly suitable for DEAE-cellulose (Fahey *et al.* 1958). The end of the syphon in *C* was deflected to prevent troublesome suction effects from the magnetic stirrer. Small variations in the gradient could be achieved by raising or lowering flask *B* relative to *C*. Replacement of *C* by a conical flask gave linear gradients suitable for CM-cellulose. The pH of the effluent was measured (with a Pye Dynacap pH meter) in most runs to ensure that undue random variation of the gradient did not occur. Flow rates were of the order of 100–200 ml./hr. Variation of flow rates had virtually no effect on results (cf. Sober & Peterson, 1958). Recoveries of u.v. absorption were in the range 95–105%.

Fig. 1. General arrangement of the column and gradient apparatus (for details see text).

Analysis of effluents. Protein in the effluent was determined from the u.v. absorption, either at 210 or 280 $m\mu$. Measurements at 210 $m\mu$ were made with a Hilger Uvispek spectrophotometer on 6 ml. fractions. At 280 $m\mu$ an automatic scanner measuring continuously on the column effluent was used. This apparatus is a development of that described by Cooke *et al.* (1959) and will be reported in detail elsewhere.

Concentration of effluent. Effluent fractions were pooled after inspection of the record and were concentrated before immunoelectrophoresis. Dialysis against polyethylene glycol (mol.wt. 20 000), polyvinylpyrrolidone (British Oxygen Co. Ltd.) and evaporation of water through a dialysis sac in a current of air at 4° (pervaporation) were tried as methods of concentration. The possible effect of contamination by polyethylene glycol and polyvinylpyrrolidone was investigated by an adaption of immunoelectrophoresis with solutions of polyethylene glycol or polyvinylpyrrolidone in the central trough instead of anti-serum. Heavy turbidity occurred in the α_2 -globulin region with polyvinylpyrrolidone, but none could be detected with polyethylene glycol. This confirmed our previous impressions that polyvinylpyrrolidone could not be safely used because it caused precipitation of α -globulins. Polyethylene glycol also contaminated the fractions during dialysis, and although it did not affect subsequent electrophoresis it did interfere with chemical characterization. Pervaporation (although much slower) was therefore used in all the experiments described here. All concentrations were carried out at 4° and thereafter most of the salts remaining in the dialysis sacs were removed by dialysis against water. Turbidity frequently developed during concentration and in some cases the proteins could not be redissolved. The degree of concentration was varied in

order to achieve a suitable final protein concentration (about 1–3 %).

RESULTS

Figs. 3–6 show the results of DEAE-cellulose chromatography of fractions obtained by starch-grain electrophoresis. Fig. 2 shows the result obtained with whole serum with the same gradient and column as for the fractions. It should be added that since the degree of concentration varied for different fractions, the immunoelectrophoresis diagrams are not quantitatively comparable, and only describe the qualitative composition of the fraction. The areas on the chromatogram give, however, a rough quantitative estimate of the different fractions. The proportion of the applied protein eluted in each fraction, obtained by area analysis, is shown in each figure. In calculating these values it has been assumed that all globulins have the same specific absorption at 280 $m\mu$ and that albumin has an absorption half that of globulins. This correction has only been applied where zone electrophoresis indicated that the fraction was predominantly albumin (i.e. for fraction e, Fig. 2; b, Fig. 5; a and b, Fig. 6; and c and d, Fig. 7).

Identifications were based mainly on the appearance and position of the immunoelectrophoretic line, with the nomenclature of Grabar & Burtin (1960). Many proteins, particularly α -globulins, detectable by immunoelectrophoresis, have not yet been named, and where a reasonably certain attribution was impossible they have been labelled merely to indicate their electrophoretic mobility.

Ouchterlony diffusion plates were used to find whether the same protein occurred in several different fractions from a single chromatogram, and these are shown below the immunoelectrophoresis results in Figs. 3 and 8.

γ -Globulin was fractionated by DEAE-cellulose chromatography into four distinct peaks (Fig. 3). All fractions were of γ -globulin mobility, but immunoelectrophoresis and Ouchterlony diffusion experiments revealed differences between them. The major component occurred in fractions a, b and c, but in c a second minor component (possibly γ_2 of Heremans, 1959) was found, whereas d was almost entirely a third component of fast γ -globulin mobility; the line due to this component showed partial fusion with the main γ -globulin line and behaved on immunoelectrophoresis like β_{2a} -globulin. Fractions e and f gave only faint lines on Ouchterlony plates and were largely denatured after concentration. They were identical with each other and with a trace component in d.

The β -globulins were split into five peaks on DEAE-cellulose chromatography (Fig. 4), though

the first one (*a*) proved to be 'γ-globulin' immunologically, *b* contained all the siderophilin and 'γ-globulin' of β-globulin mobility. In addition β_{1B}-globulin and traces of two other unidentified β-globulins were detected. β_{1B}-Globulin is the component which has been reported by Tombs (1960)

as possibly combining with haemoglobin and by Biserte, Havez, Hayem & Laturaze (1960) as combining with haemoglobin, myoglobin and cytochrome *c*. However, Aber & Rowe (1960) have reported a β-globulin that binds haematin. This may account for the apparent haem-protein

Fig. 2. Chromatography of 4 ml. of serum on DEAE-cellulose column (8 × 2 cm.) with gradient elution. The conical flask (*B*, Fig. 1) (1 l.) contained 0.3 M-sodium phosphate, pH 4.24. The round flask (*C*, Fig. 1) (500 ml.) contained 0.01 M-sodium phosphate, pH 8.38. The base of the conical flask was 7.7 cm. below the base of the round-bottomed flask. The gradient terminated at fraction 117 when flask *C* was empty. The buffer remaining in flask *B* was then allowed to flow straight through. ---, pH variation; —, u.v. absorption of the effluent. Tubes were pooled into fractions *a*–*h* corresponding to the regions of the chromatogram as shown at the top. The figures at the top are the percentages of the total eluted protein in that fraction. (For method of calculation see text.) Below is shown the result of immunoelectrophoresis of each fraction.

binding previously ascribed to β_{1B} -globulin, or may be a separate phenomenon. β_{1B} -Globulin bound haematin strongly. Peak *d* contained almost all the lipid-staining material and appeared to be mainly lipoprotein, whereas *e* contained at least two more unidentified β_2 -globulins. However, fraction *e* was largely denatured after concentration and the two lines seen were of dubious significance. The 'γ-globulin' in *a* and *b* gave a reaction of identity with the γ-globulin fraction *a* (Fig. 3).

The chromatogram of the α_2 -globulins (Fig. 5) gave an indication of the extreme heterogeneity of this fraction. There were signs of partial resolution into numerous components. Several gradients were tried, both sharper and less sharp than the one shown, but this last gave the best results. Fraction *a* contained traces of 'γ-globulin' of α_2 -globulin mobility, *b* nearly all the albumin, whereas *c* contained predominantly slower-migrating, and *d* and *e* faster-migrating, α_2 -globulins. Fraction *b* contained haptoglobins. No α_2 -lipoprotein was detected although the original α_2 -globulin fraction contained it. It was presumably lost during the manipulation.

The albumin fraction contained all the α_1 -globulins together with traces of α_2 -globulin. Fig. 6 shows the result of DEAE-cellulose chromatography with the standard gradient system.

Fig. 3. Chromatography of the γ-globulin fraction obtained by electrophoresis of 15 ml. of serum. Conditions of chromatography were as for Fig. 2. For arrangement of figure see legend to Fig. 2.

The major part of the albumin (fraction *a*) was homogeneous by immunoelectrophoresis. Peaks *b* and *c* contained the α -globulins together with trailing albumin.

Fig. 4. Chromatography of the β-globulin fraction obtained by electrophoresis of 15 ml. of serum. Conditions of chromatography were as for Fig. 2.

Fig. 5. Chromatography of α_2 -globulin fraction obtained by electrophoresis of 15 ml. of serum. Conditions of chromatography were as for Fig. 2.

On CM-cellulose the α_1 -globulins were eluted before the albumin, and could thus be obtained completely free from this component. Fig. 7 shows a typical experiment for the preparation of α_1 -globulins. In this case, however, albumin was not obtained free from other minor α -globulins. Fig. 8 shows the results of Ouchterlony diffusion experiments. These were an attempt to discover the overlap between fractions from the same chromatographic run and, in one case, fractions from different runs.

Inspection of the immunoelectrophoresis results had shown that albumin, for example, occurred in several different fractions. In the Ouchterlony plates reactions of identity, especially between components of adjacent fractions, were frequently observed and it was clear that many components, revealed by immunoelectrophoresis of a given fraction, must be immunologically identical with those found in others, particularly neighbouring ones. Selected fractions from different runs, e.g. β c and albumin c, also showed common components. It was not possible to identify most of the precipitation zones. These experiments confirm the heterogeneity found by immunoelectrophoresis.

Small-scale chromatography

Serum (0.2 ml.) was separated by paper electrophoresis, and segments corresponding to each fraction were cut out by using stained marker strips as a guide. Each segment was extracted with DEAE-cellulose-equilibrating buffer and the ex-

tract and paper segment were placed on top of a column. Chromatography was then carried out in exactly the same way as for the larger scale experiments described above, protein in the effluent being detected at 210 m μ . Owing to the slightly higher resolving power of paper electrophoresis it was possible to obtain an α_1 -globulin fraction. Veronal buffers could not be used because they absorb strongly at 210 m μ : borate does not, and appears to give electrophoretic patterns similar to veronal. The results obtained are shown in Fig. 9. They were similar to those from large-scale runs. The β -globulins did not show a siderophilin peak, although if an equivalent amount of β -globulin obtained by starch-grain electrophoresis was chromatographed the peak was present. It was concluded that this globulin could not be recovered after paper electrophoresis. The small peak appearing at the start of every run was due to material

Fig. 6. Chromatography of albumin fraction obtained by electrophoresis of 15 ml. of serum. Conditions of chromatography were as for Fig. 2.

Fig. 7. Chromatography of albumin from 12 ml. of serum on a CM-cellulose column (8 cm. $\times$ 2 cm.) with gradient elution. The conical flask (B, Fig. 1) (1 l.) contained 0.50 M-sodium acetate, pH 7.8. The round-bottomed flask (C, Fig. 1) (500 ml.) contained 0.05 M-sodium acetate, pH 4.5. Both flasks were at the same level. After applying protein to the column the equilibrating buffer from the reservoir (A, Fig. 1) was run through and was continued to fraction 32 when the gradient was begun.

extracted from the paper. The size of the peak depended on the area of paper extracted, and was absent from the runs with starch-grain fractions (e.g. β -globulin in Fig. 9).

DISCUSSION

The electrophoretic criterion is by far the most widely used for classification of serum proteins. The categories correspond fairly closely with functional subdivisions, and some modifications of electrophoresis have a high resolving power. Electrophoresis has, therefore, been our main method of judging the effectiveness of chromatographic fractionation: simple zone electrophoresis as a primary criterion and immunoelectrophoresis for finer distinctions.

It is evident from Fig. 2 and numerous examples in the literature that chromatography of whole serum fails to give resolution into electrophoretically homogeneous components. Some components, albumin in particular, were eluted from the column over a wide range of conditions.

The choice of a suitable prior fractionation method depended largely on practical considerations: a method capable of dealing with fairly large quantities of protein with minimal denaturation was required.

Ammonium sulphate fractionation is one such method but, as with column chromatography, its resolving power is poor for α - and β -globulins. A combination of these two methods gave good results with the more soluble proteins (Cooke *et al.* 1959; Tombs, James & MacLagan, 1961) but was not applicable to the remainder. Alternating chromatography on CM- and DEAE-cellulose has been used for fine resolution of γ -globulin (Sober & Peterson, 1958). It is possible to devise a procedure for the complete fractionation of serum proteins into components homogeneous by zone electrophoresis by using a combination of ammonium sulphate fractionation, CM-cellulose and DEAE-cellulose chromatography (unpublished results). This was lengthy and yields were poor, largely because of the excessive dilution of the protein and the frequent change of pH necessary.

Fig. 8. Diagrams of Ouchterlony diffusion plates showing relationships between fractions. The fractions are the same as those shown in Figs. 2-7.

An attempt to utilize the advantages of alternating CM- and DEAE-cellulose chromatography whilst avoiding the disadvantages mentioned above was also made by packing one material immediately on top of the other. The results were complex but suggested that this approach was not suitable for general fractionation, although it might find special application, for example to γ -globulin, or for the preparation of albumin.

Electrophoresis is perhaps the best method of preliminary fractionation, but has the disadvantage of limited capacity. In our experience the components were never completely separated by starch-grain electrophoresis, but the cross-contamination was probably less than in other methods and the losses were small. The use of this method as a prelude to column chromatography has not previously been reported, except for γ -globulin (Fahey & Horbett, 1959). The combination has yielded considerable additional fractionation, of which some features are notable.

Fig. 9. Chromatography on DEAE-cellulose of fractions obtained by paper electrophoresis of 0.2 ml. of serum, except for β -globulin, which was obtained by starch-grain electrophoresis. Conditions of chromatography were as for Fig. 2, but protein was detected at 210 instead of at 280 $m\mu$.

In agreement with Fahey & Horbett γ -globulin was fractionated into the main and macroglobulin components, but in addition the presence of β_{2a} -globulin was recognized. The high hexose content of β_{2a} -globulin (Heremans, Heremans & Schultze, 1959) could account for the rise in hexose content, with increasing difficulty of elution under similar conditions, observed by Fahey & Horbett (1959).

Both the α - and β -globulins were plainly heterogeneous by chromatography, particularly the α -globulins, and the chromatographic fractions themselves were still mixtures as shown by immunoelectrophoresis. But it should be emphasized that immunoelectrophoresis may give a false impression of the relative amounts of the components of such a mixture. For example, the results in Fig. 5 suggest that fraction *b* contained a high proportion of α -globulin, but simple agar electrophoresis showed that it was almost entirely albumin. Chromatography can, however, be used to give a completely homogeneous albumin by immunoelectrophoresis (fraction *a*, Fig. 6). Although the acid pH required for CM-cellulose chromatography denatured the α_2 -globulins, a group of α_1 -globulins could easily be separated from albumin, a fractionation which is difficult by any other method (Fig. 7).

The Ouchterlony diffusion experiments (Fig. 8) were an attempt to define the overlap between fractions from the same chromatographic run. It was not possible to identify most of the precipitation zones, but reactions of identity, particularly between components of adjacent fractions, were frequently observed and it was clear that many components revealed by immunoelectrophoresis of a given fraction must be immunologically identical with those found in others. Indeed, judged by this purely qualitative criterion the overall resolution was poor.

Although one of the main advantages of ion-exchange cellulose chromatography is the large amount of protein which can be handled by comparatively small columns, very small amounts of protein (less than 1 mg.) could also be successfully run on DEAE-cellulose. Although excessive trailing prevented satisfactory results on CM-cellulose, patterns similar to normal CM-cellulose runs could be obtained with cellulose phosphate on a small scale (Cooke *et al.* 1959).

The interpretation of chromatograms measured at 210 $m\mu$ is easier than that of those measured at 280 $m\mu$ as the specific absorptions of serum proteins are more nearly the same at the former wavelength. The use of this wavelength permits the combination of chromatography with methods of electrophoresis which can only deal with small quantities of protein and offers a method of assessing the composition of fractions isolated by, for example, starch-gel electrophoresis.

SUMMARY

1. Experiments are described in which normal serum proteins were examined by modified cellulose chromatography after an initial electrophoretic separation.

2. Diethylaminoethylcellulose chromatography gave useful resolution of fractions obtained by starch-grain electrophoresis. Methods for the combination of chromatography with small-scale electrophoretic methods (with 0.2 ml. of serum) are described.

3. The general application of chromatography to serum protein fractionation is discussed.

We are grateful to the British Empire Cancer Campaign and to the Endowment Fund of the Westminster Hospital for generous financial support. We also thank our colleagues who gave blood.

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Biochem. J. (1961) **80**, 292

Aromatic Biosynthesis in Higher Plants

1. PREPARATION AND PROPERTIES OF DEHYDROSHIKIMIC REDUCTASE

BY DORIS BALINSKY* AND D. D. DAVIES†

Botany Department, University of London King's College, London

(Received 7 November 1960)

Davis and his co-workers (see Davis, 1955) indicated that the pathway of biosynthesis of the aromatic amino acids in *Escherichia coli* proceeded via the key intermediates dehydroquinic acid, dehydroshikimic acid and shikimic acid. The complete pathway from glucose to phenylalanine and tyrosine has since been elucidated by the recent work of Weiss & Mingioli (1956), Schwinck & Adams (1959) and Srinivasan & Sprinson (1959). Several workers have demonstrated the incorporation of labelled shikimic acid into a number of aromatic plant constituents (Brown & Neish, 1955; Eberhardt & Schubert, 1956; Underhill, Watkin & Neish, 1957; McCalla & Neish, 1959; Kosuge & Conn, 1959).

* Present address: Metabolic Research Unit, South African Institute for Medical Research, Johannesburg, South Africa.

† Present address: C.S.I.R.O. Unit, Botany Department, Sydney University, Sydney, Australia.

The enzyme forming shikimic acid from its immediate precursor, dehydroshikimic acid, was demonstrated in cell-free extracts of *E. coli* by Yaniv & Gilvarg (1955) and named 'dehydroshikimic reductase'. This enzyme catalyses the reduction of dehydroshikimic acid, utilizing the reduced form of triphosphopyridine nucleotide as coenzyme. The enzyme was purified approximately ninefold, and some of its properties were studied. The presence of the enzyme was demonstrated in a number of organisms which can synthesize their own aromatic amino acids, namely *Aerobacter aerogenes*, *Saccharomyces cerevisiae*, *Euglena gracilis*, spinach leaves and peas.

This paper describes the extraction and purification of dehydroshikimic reductase from etiolated epicotyls of peas, and some of the properties of the purified enzyme. A preliminary report of this work has already been published (Balinsky & Davies, 1960).