ornithine transaminase system alone is not adequate as an ornithine-synthesizing mechanism *in vivo*. Possibly one of the steps in the hypothetical part of the above cycle is deficient in these mutants.

The conversion of Δ^1 -pyrroline-5-carboxylic acid to proline certainly occurs in *Esch. coli* and *Neurospora* since either of these compounds will satisfy the growth requirements of certain mutant strains of both organisms. The *Esch. coli* mutant 55-1 and the *Neurospora crassa* mutant 21863, both of which require proline specifically, are presumably unable to carry out this conversion. Taggart & Krakaur (1949) have concluded that L-proline is oxidized by the mammalian cyclophorase system by way of glutamic semialdehyde, and the reactions connecting these two compounds may be freely reversible.

SUMMARY

1. An enzyme present in extracts of *Neurospora crassa* mycelium transfers the δ -amino group of L-

ornithine to α -ketoglutaric acid with the formation of glutamic acid and glutamic γ -semialdehyde. The latter compound is partly converted, probably spontaneously, to Δ^1 -pyrroline-5-carboxylic acid, and there is some evidence that this is in enzyme-catalysed equilibrium with another closely related compound.

2. If *o*-aminobenzaldehyde is added to trap the Δ^1 -pyrrolinecarboxylic acid the reaction goes to completion; otherwise, under the conditions of the experiments, it comes to equilibrium with about 80% of the ornithine deaminated, starting with equimolar amounts of ornithine and α -ketoglutarate.

3. Evidence has been obtained for the synthesis of ornithine from glutamic γ -semialdehyde and glutamate by the same enzyme preparations.

4. Both mycelial extracts and intact mycelium of mutant strains of *Neurospora* having a growth requirement for ornithine showed a normal amount of ornithine transaminase activity.

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Partition Chromatography of Insulin and Other Proteins

By R. R. PORTER

National Institute for Medical Research, Mill Hill, London, N.W. 7

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A method for partition chromatography of ribonuclease has been described in which use is made of two-phase mixtures prepared from ammonium sulphate, ethylene glycol monoethyl ether (cellosolve) and water with kieselguhr as support for the stationary phase (Martin & Porter, 1951). Further investigation of phase mixtures of a similar type, but using other glycol ethers and a variety of inorganic and organic solutes, has shown that a considerable number of such systems can be produced. Many of these are suitable for the partitioning of proteins and in some cases they may be adapted for successful partition chromatography. This paper describes the difficulties inherent in the technique and its particular application as a method of

isolation or assay for insulin. Brief descriptions of the method have already been given (Porter, 1952*a*, *b*).

EXPERIMENTAL

Materials

All the glycol ethers used were redistilled after addition of SnCl_4 using a good fractionating column. The only criterion of purity used was that there should be minimal absorption at 280 $\text{m}\mu$.

The kieselguhr was Hyflo Super-Cel obtained from Johns-Manville Co. Ltd., Artillery Row, London, S.W. 1. It was allowed to stand overnight in 2N-HCl, washed with water until free of chloride and dried at 110°. Silane-treated

Hyflo was prepared as described by Howard & Martin (1950). All insulin samples used from the most crude to the five-times-recrystallized material were generously supplied by Boots Pure Drug Co. Ltd., Nottingham.

Methods

Protein concentration in the effluent was measured by absorption at 275 or 280 $m\mu$. in a Unicam spectrophotometer using a 1 cm. cell. An insulin solution containing 1 mg./ml. has an extinction coefficient ($\log I_0/I$), in a 1 cm. cell, of 1.0.

Insulin assay was made using the mouse convulsion test. The procedure adopted was to use four groups of twenty-four mice, one group of high and one of low dose (low dose = half high dose) for test, and two similar groups for standard. The mice were kept in glass jars in a cabinet maintained at 30°. Three such lots of ninety-six mice constituted one assay. A critical discussion of this well established technique has recently been given by Smith (1950).

Qualitative tests for the hyperglycaemic glycogenolytic factor were carried out by following the blood-sugar level in rabbits, or alternatively by the rabbit liver-slice method described by Sutherland & Cori (1948). Phase diagrams were prepared as previously described (Martin & Porter, 1951) by titration of the aqueous salt solution with organic solvent to the first sign of opalescence. It should be emphasized that these diagrams are approximate, though adequate for the purpose required.

Preparation of the column. If the organic-solvent phase is to be kept stationary, silane-treated Hyflo is used as support, and if the aqueous phase is stationary, acid-washed Hyflo is used. The only exception is the $(\text{NH}_4)_2\text{SO}_4$ -cellosolve-water system, where Hyflo is preferentially wetted by the organic phase. The reason for this exceptional behaviour is not known.

In certain systems, notably cellosolve-phosphate, the slurry obtained with silane-treated silica is very frothy and it is impossible to pack a column satisfactorily. If, however, 5–10% (v/v) of ethylene glycol monobutyl ether (butyl cellosolve) is added to the cellosolve before use, the foaming is eliminated without significant effect on the phase system.

When the column is packed, it is frequently observed that the effluent has a higher absorption at 280 $m\mu$. than would be expected. This falls off rapidly and is presumably due to the washing from the column of impurities absorbing in this range. Hence it is normal practice to allow the moving phase to run through columns for a short time before adding the protein solution.

The rate of flow of solvent through columns is not critical, but the head is usually adjusted so that no more solvent than is equivalent to half the volume of the column flows through in 1 hr.

RESULTS

Chromatography of insulin

Insulin is an exceptionally stable protein which is soluble in aqueous solutions containing high concentrations of organic solvents and yet is easily thrown out of solution by the addition of small amounts of salt. This rather unusual combination of properties makes it a simple protein to handle with this type of partition chromatography, and there is

little doubt that suitable phase systems other than those described here could also be found.

System 1. The butyl cellosolve-phosphate system (Fig. 8) was first investigated and a suitable phase mixture found which had the following composition: 83 ml. water, 45.5 ml. butyl cellosolve, 10 ml. 2.5M-potassium phosphate (pH 7.6).

In all these mixtures the insulin is more soluble in the upper phase and hence this is the stationary phase, silane-treated silica being used as support. On such a column crystalline insulin gave a single main active peak together with trace inactive peaks. When the mother liquor left after the first crystallization of insulin in the industrial preparation was chromatographed the result shown in Fig. 1 was

Fig. 1. Chromatography of protein in mother liquor from first crystallization of insulin (20 mg. dry matter). System: 83 ml. water, 45.5 ml. butyl cellosolve and 10 ml. 2.5M-potassium phosphate (pH 7.6). Column made with 10 g. silane-treated silica in tube 1.1 cm. int. diam.

obtained. This material contained about 5% of insulin and on the column this was obtained as a peak well separated from the impurities. Much impurity remained stationary at the top of the column and did not appear in the chromatogram.

The insulin travelled at $R=0.8$, and it was considered desirable to investigate its behaviour in other systems where it ran more slowly. The butyl cellosolve mixtures are rather difficult to handle because of their temperature lability.

System 2. A similar system employing phosphate at pH 7.6, but using a mixture of ethyl and butyl cellosolves, was next examined. That finally chosen was as follows: 15 ml. 2.5M-potassium phosphate (pH 7.6), 13 ml. water, 5.33 ml. ethyl cellosolve and 2.67 ml. butyl cellosolve.

This mixture is also temperature-labile, but to a much less marked degree than when butyl cellosolve alone is used. Satisfactory chromatograms may be run without special precautions to control the temperature. The partition coefficient of insulin in this system is 25:1 in favour of the upper phase and

$R=0.15$. A diagram obtained using five-times-recrystallized insulin is shown in Fig. 2.

If the efficiency of this column (6 g. silica; int. diam. 1.1 cm.) is calculated according to the formulae derived by James & Martin (1952) it is found to be equivalent to 400 plates in a counter-current extraction process, and yet, in spite of this high resolving power, the insulin peak appears to be homogeneous. It is possible that small amounts of impurities were present which were not eluted. When working with a very slow-moving peak such as this, particular care is necessary in the packing of the column to free it entirely from air bubbles and to ensure evenness. If this is not done, distortion of the peak occurs and this distortion is not, of course, reproducible from one column to the next.

Fig. 2. Chromatography of five-times-recrystallized insulin (5 mg.). System: 13 ml. water, 5.33 ml. ethyl cellosolve, 2.67 ml. butyl cellosolve, 15 ml. 2.5M-potassium phosphate (pH 7.6). Column made with 6 g. silane-treated silica in tube 1.1 cm. int. diam.

It was observed with this system and also with the acid phosphate system to be described, that slight stripping of the stationary phase from the column occurred. This is possibly associated with the temperature lability of these systems, but it is so slow as to have no effect on the chromatogram. If, however, the same column was used a second time the R values of the insulin and impurities increased, indicating that partition was being effected over a portion of the column only, usually 70–90% of the full length.

System 3. In a further attempt to resolve the insulin peak a system employing mixed ethyl and butyl cellosolves but using sodium dihydrogen phosphate and phosphoric acid to give pH 2.5 or 3 was devised. Such a system has the additional advantage that if crude pancreatic extracts are being handled there is less risk of proteolytic degradation at an acid pH. If large-scale preparative work were envisaged with such a system it would be preferable to correlate the rate of loss of insulin with the pH of the pancreatic extract and choose that in which the loss was minimal.

Fig. 3. Chromatography of crude insulin A (10 mg.). System: 15 ml. water, 6.67 ml. ethyl cellosolve, 3.33 ml. butyl cellosolve, 9 ml. 5M- NaH_2PO_4 (+ H_3PO_4 to pH 3). Column made with 6 g. silane-treated silica in tube 1.1 cm. int. diam.

Fig. 4. Chromatography of crude insulin B (10 mg.). Solvent system and column as in Fig. 3.

Fig. 5. Chromatography of crude insulin C (100 mg.). Solvent system as in Fig. 3. 50 g. silane-treated silica in tube 2.3 cm. int. diam.

The mixture chosen was as follows: 9 ml. 5M-sodium dihydrogen phosphate adjusted by addition of phosphoric acid to pH 3, 6.67 ml. ethyl cellosolve, 3.33 ml. butyl cellosolve, 15 ml. water.

Using this system a series of crude insulin preparations obtained from different stages in the industrial preparation was examined. The first step in insulin manufacture is the extraction with acidified ethanol of minced pancreas. The materials chromatographed were the total protein from the second (crude insulin *A*), the third (crude insulin *B*) and the fourth (crude insulin *C*) steps in the process. As received, crude insulin *A* contained 70% of salt, which was removed by dialysis against 0.01N-hydrochloric acid at 2°, the bag contents being freeze-dried. The chromatogram obtained with this material is shown in Fig. 3. In all three solvent systems much inactive material remained on top of the column, presumably because it was either insoluble in the moving phases or had a very high partition coefficient in favour of the stationary phases. The insulin content of fraction *A*, calculated from the area of the insulin peak, was 8.5 or 2.8% of the total sample before removal of salt by dialysis.

Fig. 4 shows the chromatogram obtained with the crude insulin *B*. No dialysis was necessary and this sample was found to contain 12.5% of insulin. Fig. 5 shows the result with crude insulin *C* and there is now 37% of insulin. It can be seen that from step 3 to step 4 the fast-running impurity has been eliminated.

The great difference between the rate of movement of the insulin ($R=0.38$) and all impurities which were stationary or ran fast indicates the high selectivity of this system. For preparative use, mishandling of the column, due to poor packing, overloading or pronounced temperature changes, does not prevent the isolation of a pure product, though the insulin peak will of course be spread or distorted.

Biological assay of insulin peaks. Qualitative tests for insulin activity in the different fractions from the columns were made by injecting twelve mice according to the method described and at increasing concentrations. In all tests, convulsions were produced only when the main insulin peak was used and there was no evidence of hypoglycaemic activity among the other fractions.

A number of assays were made on the insulin peak and the results obtained with a series of crude insulins obtained by fractionation with system 3 are given in Table 1. For these assays the effluent fractions corresponding to the whole peak were collected, pressure-dialysed and freeze-dried. For preparative work precipitation of the insulin by adjusting the pH to 5 would be preferable, but for the present purpose it was desired to avoid any possibility of purification subsequent to collection from the column. Similar assay results were obtained if the effluent was used directly, as the dilution required is so great as to eliminate undesirable effects of the solvent and salt in the lower phase.

It would appear that in all cases the insulin obtained from the column had an activity of about 24 i.u./mg. and hence was of similar activity to the International Standard 1952 (Miles, Musset & Perry, 1952).

Table 1. *Assay of insulin peaks from columns*

Material used	Insulin activity found (i.u./mg.)	
	Mean	Range
Crude insulin <i>A</i>	24	18-29
Crude insulin <i>B</i>	25	20-30
Crude insulin <i>C</i>	26	20-32
Crude insulin <i>C</i> *	23.8	21.4-26.2

* Assay by Burroughs Wellcome.

Recovery of insulin from the column. Direct comparison of the insulin content of crude samples as estimated by this technique with that found by biological methods is not possible since biological assay is unsatisfactory when the test material is of a much lower potency than the standard. However, the weight recovered when pure insulins are fractionated and the increase in size of the peak when pure insulin is added to the crudest material available have been measured. Pure insulin, both a five-times-recrystallized sample, and material purified on the column have been used, and it can be seen from Table 2 that they give slightly different results.

Table 2. *Recovery of insulin from columns*

(Estimation by absorption at 275 m μ . Solvent system 3. 6 g. of silane-treated Hyflo were used in a tube of 1.1 cm. int. diam.)

Material used	Recovery of added insulin (%)
Crystalline insulin (5 mg.)	81
Crystalline insulin (5 mg.) + crude insulin (10 mg.)	86
Chromatographically purified insulin (5 mg.)	97
Chromatographically purified insulin (5 mg.) + crude insulin (10 mg.)	92

With insulin previously purified chromatographically the recovery is near 100%, but with crystalline insulin it is some 10% less. This is possibly due to small amounts of impurity in the latter, as at least 5% could be present without significant effect on the biological assay, in which it is difficult to reduce the error below 10%.

Further confirmation of the nearly quantitative recovery of insulin from these chromatograms was obtained from the insulin content of the three crude samples, described earlier. Estimation by the manufacturers, based on calculation of the yield finally obtained, was in good agreement with that found chromatographically.

The hyperglycaemic-glycogenolytic factor. When tested using the rabbit liver-slice method (Sutherland & Cori, 1948), the insulin isolated from the column showed little glycogenolytic activity when 300 μ g. were used in each assay. It will be observed, however, in Fig. 5, where the chromatogram obtained when 100 mg. of crude insulin have been run on a 50 g. column is shown, that there is a small peak (about 1% of the area of the insulin) running faster ($R=0.5$) than the insulin ($R=0.38$). This material was collected, pressure-dialysed, freeze-dried and tested for glycogenolytic activity. Although the assay could not be reduced to a satisfactory quantitative basis, it was apparent in parallel experiments that this minor component was some 100 times more effective than insulin in stimulating glycogen breakdown. It is possible that this small peak represents the pure hyperglycaemic-glycogenolytic factor, though isolation in weighable amounts and more careful study will be necessary to establish this.

*Anomalous chromatographic behaviour
observed with certain proteins*

Although insulin behaved throughout in a satisfactory manner when fractionated chromatographically, work with other proteins has shown that anomalous behaviour is occasionally met.

Ribonuclease. It has previously been reported (Martin & Porter, 1951) that beef pancreatic ribonuclease when chromatographed in the ammonium sulphate-cellosolve system showed one main peak and one small peak, both of which were enzymically active. It has since been observed that ribonuclease from pig pancreas shows only one such peak, whilst in that from foetal calf pancreas the minor component is present only in trace amounts.

During partial-hydrolysis studies of beef pancreatic ribonuclease, it was found that after the addition of pepsin or carboxypeptidase (Fig. 6) new enzymically active peaks appeared. The proteolytic enzymes were added in approximately 1% of the weight of ribonuclease. These new peaks were first considered to be a degradation product of the ribonuclease which retained activity, but this view had to be abandoned for the following reasons:

(1) The original enzyme has one *N*-terminal lysine residue as estimated with fluorodinitrobenzene. The new compounds isolated after treatment with pepsin or carboxypeptidase again show only lysine as *N*-terminal residue but in significantly smaller quantity.

(2) With both proteolytic enzymes the formation of the new compounds is very rapid at a pH unfavourable to digestion. Thus carboxypeptidase and ribonuclease may be dissolved in the lower phase (pH 5) and immediately run on a column. The main enzymically active peak again travels at a

characteristically slower rate than the original and there is no trace of the original peak. The rate of movement of the small peak is unaltered.

(3) When the carboxypeptidase-treated enzyme from the column was collected and dialysed over several days a precipitate eventually appeared which had carboxypeptidase activity. It is presumed that digestion had continued as far as possible and that the carboxypeptidase then dissociated, and, owing to its insolubility in salt-free water, precipitated.

Fig. 6. Chromatography of ribonuclease. System: 20 g. $(\text{NH}_4)_2\text{SO}_4$, 20 ml. ethyl cellosolve and 56 ml. water. 6 g. silica in tube 1.1 cm. int. diam. (a) 5 mg. beef ribonuclease. (b) 7 mg. beef ribonuclease + 0.1 mg. carboxypeptidase.

This would appear to be an example of an enzyme-substrate complex which in chromatographic behaviour simulated a single component.

Bovine serum albumin and penicillinase. These proteins could be chromatographed in an apparently satisfactory manner, but on further examination showed anomalous behaviour which has not been satisfactorily explained. Crystalline bovine serum albumin when fractionated using phase mixtures prepared from water, potassium phosphate (pH 7.6) and ethyl and butyl cellosolves revealed a main double peak together with trace components moving rapidly. The main peaks were never fully resolved, and their relative size changed considerably from one chromatogram to the next. Crude penicillinase was the freeze-dried culture filtrate from *Bacillus cereus* which had been grown on casein hydrolysate to which 1% gelatin had been added in order to stabilize the enzyme. This material could be fractionated successfully using phase mixtures of

ammonium sulphate, water and ethyl cellosolve at 2°. When the enzymic activity of the effluent fractions was assayed, a sharp single peak was found, the specific activity of which was 10–15 times greater than that of the original material. However, the rate of movement of the enzymically active material varied from $R=0.3$ to $R=0.6$ for different enzyme preparations, though the same phase system was used. It is possible that in both cases some type of association-dissociation phenomenon was responsible for the erratic behaviour.

DISCUSSION

Choice of phase mixture

The choice of phase mixture when applying this type of chromatography to a protein is influenced by two major difficulties which have to be overcome.

(1) *Denaturation of the protein.* The risk of denaturation arises from two causes.

(a) *Denaturation by organic solvent.* Though there are notable exceptions, most proteins are denatured when organic solvents are added to their aqueous solution in sufficient concentration and at room temperature. However, it has been observed that glycol ethers, as a class, have relatively little denaturing power with all the proteins examined. For example, human γ -globulin will remain in solution in 50% (v/v) aqueous cellosolve at room temperature for some days without any apparent decrease in solubility at its isoelectric point. There will no doubt be exceptions to such behaviour and if met they may be avoided by working at -5° . The change in temperature may alter the phase diagram of the system, but in few cases has the stability of the system been found to change. Hence, denaturation by the organic solvent should rarely be met and should be avoidable if it does occur.

(b) *Surface denaturation of the protein following adsorption.* In this type of system there are three interfaces at which the protein could be adsorbed with the possibility of subsequent denaturation. These are the solid-liquid interfaces between the stationary phase or the moving phase and the supporting kieselguhr, and the liquid-liquid interface between the stationary and moving phases. If adsorption at the solid-stationary phase interface occurred it should be readily detected by allowing a solution of the protein in the stationary phase to run through a column prepared with this phase alone. In cases where this has been done the protein has always appeared at the liquid front in the eluate indicating that adsorption at the solid-liquid interface did not occur.

Test for adsorption at the liquid-liquid or solid-moving phase interfaces can be made by comparing the rate of movement of the protein down the column with the rate calculated from the partition

coefficient of the system according to the formulae of Martin & Synge (1941). In the case of insulin with the phase system described, the agreement between the found and calculated values was good. With ribonuclease and most other proteins examined the rate of movement found was significantly lower than the calculated value and hence adsorption at one or both of these interfaces is probably occurring. Increasing the salt concentration of the system increases the adsorption sometimes to a very marked degree. Thus when rabbit γ -globulin was partitioned between a system composed of diethylene glycol diethyl ether (diethyl carbitol), magnesium sulphate and water to give a partition coefficient of 3:1, the protein had an R value (0.7) close to that calculated (0.75). However, if the partition coefficient was raised to 5:1 by moving away from the plait point, it was found that the rate of movement of the protein on the column dropped sharply and only half the material put on was recoverable in the eluate. It is probable that the increased salt concentration had greatly increased the adsorption at the liquid-liquid interface and that in this case adsorption had been followed by denaturation. A somewhat similar phenomenon was observed with crotoxin (Fraenkel-Conrat and Porter, unpublished) where a very small increase in salt concentration (1–2%) of the system changed the chromatographic pattern from a sharp peak to a long trail. In this case denaturation was confirmed by the loss of the toxicity of the protein to mice. Crotoxin is relatively stable to most common methods of denaturation, and it appears that occurrence of adsorption is as unpredictable as is the denaturation which occasionally follows. If adsorption is not followed by denaturation it may well contribute to the resolving power of the system.

(2) *Choice of phase system with a suitable partition coefficient.* Another difficulty of the method is the choice of a phase system which will give a sufficiently large partition coefficient and yet maintain a high enough solubility of the protein in the phase in which it is least soluble (i.e. the moving phase). It is desirable that a protein should be examined on columns using a variety of phase mixtures in which the rate of movement varies considerably, and in practice greater resolving power appears to be associated with lower values of R (say 0.1–0.4). Such values necessitate a partition coefficient of 10:1–40:1 if no adsorption is occurring (Martin & Synge, 1941). If the protein concentration in the eluate is to be followed by measurement of absorption at 280 $m\mu$, it is necessary that the protein should have a minimum solubility of 1 mg./ml. These two requirements are often incompatible, and only trial and error can establish the most satisfactory systems to use. There are of course some systems, the use of which for certain proteins can be

immediately excluded. Thus, in the cellosolve ammonium sulphate-water system the concentration of salt in the lower phase always exceeds 20%, and it therefore cannot be used for γ -globulin which is insoluble in such a salt concentration.

A considerable variety of phase systems have now been studied and three distinct types found, examples of which are shown in Figs. 7-9. It is apparent that the phosphate-cellosolve system could be used only for proteins soluble in strong salt solution. As sodium dihydrogen phosphate and potassium monohydrogen phosphate give similar phase diagrams it is possible to work with a stable

glycol monomethyl ether acetate (methyl cellosolve acetate).

Many different solutes have been investigated which, in sufficient concentration, cause two phases to be produced when added to water and one of the solvents mentioned. Among the inorganic salts a

Fig. 7. Phase diagram of the system NaH_2PO_4 , water and ethyl cellosolve at 18° . Arrow indicates position of plait point.

pH over a wide range. The system with phosphate and butyl cellosolve is suitable for proteins soluble in solutions with a high concentration of organic solvent. As the plait point occurs in the flat part of the curve small changes in composition greatly alter the composition of the two phases. All systems containing butyl cellosolve are temperature-labile, and control of the room temperature within 1 or 2° is essential.

The magnesium sulphate-diethyl carbitol system is most suitable for globulin type proteins which are easily thrown out of solution by organic solvent or salt. The salting-out effect may be decreased by the use of salts such as sodium chloride or calcium chloride (see Edsall, 1947). Such salts, however, are less effective in producing two phases with the glycol ethers and water.

Other organic solvents which have been used successfully in these systems are: diethylene glycol monobutyl ether (butyl carbitol), diethylene glycol monoethyl ether acetate (carbitol acetate), ethylene

Fig. 8. Phase diagram of the system K_2HPO_4 - KH_2PO_4 (pH 7.6), water and butyl cellosolve at 21° .

Fig. 9. Phase diagram of the system MgSO_4 , water and diethyl carbitol at 18° . Arrow indicates position of plait point.

high water solubility is essential or they are thrown out of solution and two phases are not produced. Of the organic solutes only those with a very low ethanol and high water solubility can be used. Examples are sucrose, glycine and other amino-acids, and the salts of organic acids such as calcium or barium acetate and formate.

Mixtures of the solvents or solutes may be made to give phase systems with intermediary characteristics. Solvents such as acetone, which absorb strongly in the ultraviolet, make the measurement of protein concentration more difficult. The glycol ethers, as purchased, frequently absorb in this range owing to impurities, and it is necessary to redistil (after addition of a reducing agent to remove peroxides). If this purification is not done, some proteins appear to adsorb these impurities, as shown by a decrease in the protein-absorption ratio at 280–260 $m\mu$.

The range of the method

It appears that the phase mixtures obtained with the glycol ethers, water and various inorganic and organic solutes can be used successfully to partition a considerable range of proteins. The principal reason for the success of these systems is that they can give rise to immiscible phases both of which have a high water content. In the diethyl carbitol-magnesium sulphate system (Fig. 9) near the plait point both phases contain more than 75% water. If, instead of chromatography, counter-current distribution was used, some of the difficulties which have been met could probably be avoided, but others such as the very slow separation of the phases when close to the plait point would probably arise. If chromatography is successful the effort required to achieve comparable resolving power is much less than in counter-current methods.

The following proteins have been chromatographed using these systems, though in some cases detailed examination has not been made: ribonuclease from beef, pig and foetal calf pancreas, avidin (Fraenkel-Conrat & Porter, unpublished), chymotrypsinogen, α and other chymotrypsins, trypsin, trypsin inhibitor and the complex (M. Green, private communication), penicillinase from *B. cereus*, bovine serum albumin. γ -Globulin has been used but with only limited success and preliminary attempts with crotoxin were unsuccessful. When avidin was used the deoxypentosenucleic acid present (Fraenkel-Conrat, Ward, Snell & Ducay, 1950) was separated from the protein, each travelling as well separated peaks. Indiffusible commercial preparations of yeast nucleic acids were subsequently found to give satisfactory chromatograms showing several components.

It is probable that this technique could be extended to a variety of proteins, though the choice of a satisfactory phase system, which is largely a matter of trial and error, may be tedious. If a new protein is to be examined, it is desirable to know its pH stability and the concentration of salt and organic solvent which precipitate it from solution. These data are a useful guide in choice of the best system, but as unpredictable factors are also involved they

may in themselves be insufficient. In general, a system giving a partition coefficient of between 5:1 and 10:1 has been found before chromatography has been attempted. If a crude mixture is used, this partition is determined by measurement of the biological activity in each phase.

In following the protein concentration in the effluent, measurement of the absorption at 275 or 280 $m\mu$ is the most satisfactory, as no material is lost and the same effluent fraction is available for subsequent biological assay or isolation of the protein. This method is limited in that it depends on a sufficient content of the aromatic amino-acids in the protein examined and, particularly in work with partial hydrolysates, colorimetric and other methods would be needed. The high content of salt and solvent might cause difficulty in estimation and isolation, particularly if working with diffusible material, though they could possibly be removed by adsorption of the protein or polypeptide on carbon or other material. Tyrosine may be chromatographed successfully using ammonium sulphate, cellosolve and water. This may be taken as an indication that peptides could also be fractionated by this technique.

Homogeneity of insulin

Harfenist & Craig (1951) have reported that a sample of insulin (activity 27 i.u./mg.) when examined by counter-current distribution between butanol and dichloroacetic acid showed two main peaks. A train of 220 equilibration cells produced a partial separation of the peaks, both of which had slightly lower activity than the original (26 and 22 i.u./mg.). In the present work no evidence of such heterogeneity was observed, though the resolving power was equal or higher and three systems at acid and alkaline pH were used. It is particularly interesting that the crudest material still showed only one peak, suggesting that different active components have not been eliminated in the preparation. From the results with ribonuclease it is, however, possible that a protein complex could behave on several columns as a single component. Whatever the resolving power of this or any other method of testing protein homogeneity, the possibility will remain that under different circumstances the same protein may dissociate to give similar or dissimilar products. The biological assay of the insulin isolated from the column is as high as that reported elsewhere, and it may be concluded that under these conditions insulin behaves as a single entity.

Use of chromatography for insulin assay

From the figures given in Tables 1 and 2 it appears that all preparations used gave insulin components of consistent purity and that the yield is near 100%.

This method could therefore be used as a simple and rapid method of insulin assay, measurement of the area under the peak giving the insulin content of the sample used. In addition to speed and simplicity this method has an advantage over biological assay in that it appears to be as satisfactory for crude as for pure insulin. It would be equally possible to use this technique for preparative work, as a single step appears to be sufficient to obtain pure insulin from crude material. The maximum weight used with these columns so far has been 150 mg. crude insulin on a 50 g. column, but there is no evidence that this loading may not be raised.

SUMMARY

1. The preparation is described of liquid two-phase systems using a variety of glycol ethers, water and organic and inorganic solutes. These systems have been found to be suitable for partitioning proteins.

2. Three such systems containing water, ethyl and butyl cellosolves and sodium or potassium phosphate have been used for the partition chromatography of insulin.

3. The insulin thus prepared from crude or crystalline material was of uniform activity of about 24 i.u./mg. The recovery from the columns appeared to be near 100%.

4. Throughout these chromatographic studies the insulin behaved as a single component.

5. The inherent difficulties of the method and the range of applicability to other proteins are discussed.

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The Isolation and some Chemical Properties of Viridicatin, a Metabolic Product of *Penicillium viridicatum* Westling

By K. G. CUNNINGHAM AND G. G. FREEMAN

Imperial Chemical Industries Limited, Nobel Division, Research Department, Stevenston, Ayrshire

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Penicillium viridicatum was first described by Westling (1911); it was classified by Raper & Thom (1949) as a member of the *P. viridicatum* series, one of the features of which is the production of a strong, penetrating, mouldy or earthy odour. Smith (1942) states that it is often impossible to decide definitely whether a given strain should be called *P. cyclopium* or *P. viridicatum*, and suggests that 'the best way of dealing with this group is to lump together all the blue-green and yellow-green strains with similar morphology as the *P. cyclopium-viridicatum* series'. The principal strain used in this work, our catalogue

number F22 and of unknown origin, corresponded closely in its characteristics with those described by Raper & Thom (1949) for *P. viridicatum* Westling. Members of the *P. viridicatum* series are of frequent occurrence upon decaying vegetation in contact with the soil. There are a number of reports of their isolation from stored grains. Koehler (1938) reported *P. viridicatum* capable of growing upon shelled maize at a moisture level of 17.6%. Semeniuk & Barre (1944) found *P. viridicatum* to be the chief mould present on maize stored in steel bins in Iowa. Marchionatto (1942), working in Buenos