administered pregnane-3 α :20 α -diol in *in vivo* experiments on human subjects. On the other hand, if the enzyme system is a dehydrogenase, then its *in vivo* function might rather be that of a 'reductase' playing some role in the formation of pregnane-3 α :20 α -diol and of other reduction products from progesterone.

SUMMARY

1. A method is described for the determination of small quantities of pregnanediol in incubated liver preparations.

2. The application of this method to the study of the *in vitro* metabolism of pregnanediol by rat and

rabbit liver has indicated that these tissues contain an enzyme or enzymes which metabolize this steroid to as yet unknown substances. This metabolism is inhibited in nitrogen but not by cyanide. The evidence presented indicates that processes of conjugation are not involved.

The authors are indebted to the Medical Research Council for a grant out of which the expenses of this work were largely defrayed; to Mr A. L. Bacharach of the Glaxo Laboratories and to Dr D. P. Cuthbertson of the Rowett Research Institute for many of the rats used in this work; to Mr T. I. Kyle who prepared the pregnane-3 α :20 α -diol; to Dr J. W. Minnis for the micro-analysis and to Mrs S. M. Atherden for skilled technical assistance.

REFERENCES

- Bernheim, F. & Bernheim, M. C. L. (1943). *J. Pharmacol.* **78**, 394.
 Cohen, P. P. (1945). In Umbreit, W. W., Burris, R. H. & Stauffer, F. J., *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, p. 75. Minneapolis: Burgess Publishing Co.
 De Meio, R. H. & Arnolt, R. I. (1944). *J. biol. Chem.* **156**, 577.
 Deutsch, W. (1936). *J. Physiol.* **87**, 56 P.
 Fieser, L. F. & Fieser, M. (1949). *Natural Products Related to Phenanthrene*, 3rd ed., p. 474. New York: Reinhold.
 Klyne, W. (1948). *Biochem. J.* **43**, 611.
 Klyne, W., Schachter, B. & Marrian, G. F. (1948). *Biochem. J.* **43**, 231.
 Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
 Levvy, G. A. & Storey, I. D. E. (1949). *Biochem. J.* **44**, 295.
 Lipschitz, W. L. & Bueding, E. (1939). *J. biol. Chem.* **129**, 333.
 Marrian, G. F. (1949). *Recent Progress in Hormone Research*, **4**, 1. New York: Academic Press.
 Pearlman, W. H. & De Meio, R. H. (1949). *J. biol. Chem.* **179**, 114.
 Schneider, J. J. & Mason, H. L. (1948a). *J. biol. Chem.* **172**, 771.
 Schneider, J. J. & Mason, H. L. (1948b). *J. biol. Chem.* **175**, 231.
 Sommerville, I. F., Gough, N. & Marrian, G. F. (1948). *J. Endocrinol.* **5**, 247.
 Sommerville, I. F. & Marrian, G. F. (1950a). *Biochem. J.* **46**, 285.
 Sommerville, I. F. & Marrian, G. F. (1950b). *Biochem. J.* **46**, 290.
 Storey, I. D. E. (1950). *Biochem. J.* **47**, 212.
 Sutherland, E. S. & Marrian, G. F. (1947). *Biochem. J.* **41**, 193.
 Verly, W. G., Sommerville, I. F. & Marrian, G. F. (1950). *Biochem. J.* **46**, 186.

Studies in Dialysis

1. AN APPLICATION OF ION-EXCHANGE RESINS

By G. A. GILBERT AND A. J. SWALLOW
Department of Chemistry, University of Birmingham

(Received 24 April 1950)

Ion-exchange resins have been used in many instances where it has been necessary to remove ions from solutions of uncharged molecules, and it was thought possible that the same methods might serve for charged molecules provided these were too large to penetrate the resin particles. The use of ion-exchange resins would then be an alternative to normal dialysis.

Experiments were carried out to see if proteins could be 'dialysed' in this way, and it was found that egg albumin could be freed from ions by passing it

through columns of cation- and anion-exchange resins without significant loss. The non-absorption of proteins by resins has been noted by other authors and applied to the fractionation of blood serum by Reid & Jones (1949). An upper limit of about 20 Å. has been suggested by Richardson (1949) for the pore size of certain commercial resins. The non-absorption of a protein by resins and its recovery in a soluble form is not a proof that minor changes have not occurred in it, and so more sensitive tests were carried out using enzymes. Urease, α - and

β -amylase and *Q*-enzyme were found to be partly or completely inactivated irreversibly, and only pepsin, which is an enzyme particularly stable to acid, passed through the resins unchanged. Exposure to acid in the column of cation-exchange resin was thought to be responsible for the loss of activity, and to avoid this the cation and anion-exchange resins were mixed together and the proteins passed through the mixture. Not much improvement was noted, urease still being completely inactivated and amylase half inactivated.

From these results it seemed clear that the method could be used for only a few enzymes, and could certainly not be used generally for native proteins. The more usual arrangement for dialysis was therefore adopted, except that mixed resins were used for removing salt dialysing through a cellophan membrane. Rather unexpectedly, when a test was made with urease, this enzyme was precipitated and inactivated. It was found that a rapid fall in pH was occurring, and in order to overcome this obstacle, a survey of the pH changes associated with dialysis was carried out. The results obtained with resins are discussed in this paper, and those concerned with ordinary dialysis against water, which are of interest for the dialysis of proteins, are given in the following paper.

METHODS

Ion-exchange resins. The cation-exchange resins used were Zeo-Karb H.I.P. or Zeo-Karb H.I., and the anion-exchange resins De-Acidite B or C, manufactured by the Permutit Company Ltd., London. The makers' instructions for regeneration were followed except when very complete purification was necessary, in which case electro-dialysis was used to remove the last traces of ionic impurity. This was accomplished by packing washed regenerated resin into a U-tube and applying 100 V. d.c. overnight, with flowing distilled water to remove the ions accumulating near the platinum electrodes.

The regeneration of mixed resins. After they have been used to absorb salt, mixed resins can be partially regenerated by washing with ammonia solution to remove absorbed salt, followed by heating at 100° under reduced pressure to remove the absorbed NH_3 . De-Acidite was completely regenerated in this way, but Zeo-Karb retained half of the absorbed NH_3 after 4.5 hr. heating. Electrodialysis was ineffective, as was regeneration with acetic acid followed by the attempted removal of the acetic acid by solvents or distillation. The ordinary methods of regeneration become available, however, if the resins can be separated. It was found that Zeo-Karb H.I.P. and De-Acidite C can be separated by making use of their difference in density. If a mixture of them was added to a saturated solution of sodium nitrate Zeo-Karb H.I.P. sank and De-Acidite C floated. De-Acidite B has the same density as Zeo-Karb H.I.P. and was, therefore, not used in mixtures, although its anion-binding capacity is greater.

Measurement of salt concentration. A column containing 7 ml. of Zeo-Karb H.I.P. was prepared in a glass tube

1.14 cm. in diameter fitted with a tap at the bottom. To carry out an estimation of the salt in a solution, the resin was washed with distilled water until the pH of the effluent was above 4.8. The column was then drained until the meniscus of the water was level with the top of the resin, after which 2 ml. of salt solution were pipetted in and allowed to seep through slowly, followed by distilled water. The first 4 ml. of effluent were discarded and the next 10 ml. collected. These contained the whole of the acid generated by exchanging the cations of the salt for hydrogen ions, and were titrated with 0.01 N-NaOH using a glass electrode. By standardization against known salt solutions, the method could be made to give results accurate to $\pm 1\%$ for solutions ranging in concentration from 10^{-2} to 10^{-4} N.

The preparation of resin cellophan. Cellophan was boiled for 1 min. in 10% (w/v) aqueous *m*-phenylenediamine and allowed to cool in the solution for 45 min. It was removed and placed for 30 min. in a 50 : 50 (by vol.) mixture of 40% (w/v) formaldehyde and 10 N-HCl at 0°. The cellophan was then washed under the tap and any resin formed on the surface during the last stage was rubbed off. The cellophan was next immersed in 0.5% (w/v) potassium dichromate solution containing 0.15 N-HCl for 30 min. After a further 30 min. in 1 N-ammonia it was washed with water. A membrane prepared in this way retained its electropositive properties for about a month provided it was not left in contact with very acid solutions.

The apparatus used for dialysis against mixed resins is shown in Fig. 1 and is self-explanatory.

Fig. 1. Apparatus for dialysis against mixtures of ion-exchange resins.

RESULTS

A urease solution, about 0.001 M in salt, was placed in a cellophan tube supported in a beaker containing water and mixed resins (equal volumes of Zeo-Karb H.I. and De-Acidite B). In 15 min. the activity of the urease had fallen to 25% of its initial value and in 6 hr. to zero. A cloudiness noted after about 15 min. had developed into a distinct precipitate after 6 hr., by which time the pH had dropped to 3.73. Under similar conditions the pH of a salt solution (0.001 M-potassium chloride) fell to 3.27.

Dialysis of 0.01 M-potassium chloride was carried out against flowing distilled water in an apparatus similar to that for 'resin dialysis' (Fig. 1). The pH fell from 5.4 to 5.0 within 10 min., and then remained constant for at least 3 hr. Dialysis of a

similar solution against flowing hydrochloric acid (pH 4.76) led to the curve shown in Fig. 2.

Fig. 2. The pH change during the dialysis of 0.01 M-KCl against dilute HCl of pH 4.76.

Fig. 3. The pH change during the dialysis of 0.001 M- (A) and 0.01 M- (B) KCl against a mixture of anion- and cation-exchange resins.

Fig. 4. The pH change during the dialysis of 0.001 M- (A) and 0.01 M- (B) KCl against mixed resins separated from the cellophan membrane by filter cloth.

Dialysis of 0.01 M- and 0.001 M-potassium chloride against resin mixtures (3 vol. De-Acidite C to 1 vol. Zeo-Karb H.I.P.) gave the results shown in Fig. 3. The effect of keeping the resins about 1 mm. away

from the cellophan by interposing a layer of filter cloth is seen from Fig. 4.

A central column of Zeo-Karb H.I.P. was held in place by a cylinder of copper gauze, and the space between this and the membrane filled with De-Acidite C. In this apparatus the pH of 0.01 M-potassium chloride increased gradually from 5.7 to 6.2 in 3 hr.

Fig. 5. The pH change of 0.001 M-KCl (A), 0.001 M-K₂SO₄ (B), and 0.01 M-KCl (C) during dialysis against mixed resins through a resin-treated membrane.

Fig. 6. The dialysis of solutions 0.001 N with respect to cations against mixed resins using a resin-treated membrane. Phosphate buffer (KH₂PO₄ + NaOH) (A), phthalate buffer (KH phthalate + NaOH) (B) and (NH₄)₂SO₄ (C).

0.0167 N-sodium hydroxide (100 ml.) was dialysed overnight in the apparatus of Fig. 1. Titration showed that 87 % of the alkali had been removed. After very thorough washing of the resin mixture in distilled water to remove excess alkali, 0.01 M-potassium chloride was dialysed. In 2 hr. the pH rose from 5.7 to 6.4.

Modification of the membrane. Cellophan was impregnated with the following basic substances: aluminium, ferric and thorium hydroxides, crystal violet, Victoria Pure Blue B.O.S., Rhodamine B.S., methyl green, Victoria Green, aniline black, paramine brown, *m*-phenylenediamine-formaldehyde

resin, melamine-formaldehyde resin, hexamethylene-formaldehyde resin alone and copolymerized with *m*-phenylenediamine or aniline. The membrane prepared using *m*-phenylenediamine was the best for dialysis. Its ability to prevent a fall in pH in dialysis against distilled water is illustrated in the following paper. Its effect during dialysis against mixed resins is shown for a number of salts in Figs. 5 and 6.

Dialysis of α -amylase. Saliva, diluted twenty times and centrifuged, was dialysed against mixed resins through resin cellophan for 48 hr. Its activity was tested with salt-free starch in the presence of

Fig. 7. The activity of α -amylase after dialysis against mixed resins through a resin-treated membrane. —○—, before dialysis; —×—, after dialysis; —□—, after adding salt to the dialysed enzyme (see text).

0.0014M-phosphate buffer ($\text{KH}_2\text{PO}_4 + \text{NaOH}$) of pH 7.12 by the method of McCready & Hassid (1943). The activity was next determined with 0.01M-sodium chloride present as well as the buffer. The results in Fig. 7 show that the enzyme was completely deactivated by dialysis and that its initial activity was almost completely restored by the addition of salt.

DISCUSSION

The observation that the pH of a salt solution, upon dialysis against resins, falls even more than that of a urease solution containing salt, indicated that the explanation of the effect was to be sought in the presence of the salt rather than of the protein. The investigation of methods of eliminating this fall were therefore confined in the main to pure salt solutions.

Three factors likely to contribute to the effect were examined, namely, (1) the selective ion-permeability of the membrane, (2) local effects due to the contact of the resins with the membrane, (3) the acidity of the water directly in contact with the resins.

The first factor was immediately indicated by the

result of the dialysis of 0.001M-potassium chloride against flowing distilled water, of pH 5.4 since it was in equilibrium with the carbon dioxide of the air. The pH of the solution fell to 0.4 unit below that of the flowing water, and remained constant for several hours (see following paper).

The factor thought likely to contribute most to the effect was the production of acid by the cation-exchange resin immediately adjacent to the membrane. This acid could penetrate the membrane before it could be absorbed by the basic resin. Evidence for this was obtained by separating the resins from the membrane. The pH fall was then eliminated for 0.001M-potassium chloride and somewhat reduced for 0.01M-potassium chloride (Fig. 4).

In addition to the local production of acid, there was the fact that the equilibrium pH of the water bathing the resins was usually about 4.8, except with freshly mixed resins. In order to gauge this effect, 0.001M-potassium chloride was dialysed against flowing hydrochloric acid of pH 4.76. The pH of the salt solution fell to 0.6 unit below this value (Fig. 2) showing that the 'resting' pH of the resins was important, and confirming that the selective ion-permeability of the membrane was contributing to the fall.

The pH was prevented from falling by two methods: (a) by interposing a layer of anion exchange resin between the resin mixture and the membrane, and (b) by pretreating the system with sodium hydroxide solution, and washing before introducing the solution to be dialysed.

A third method, which in some respects is more convenient, made use of a positively charged membrane in combination with mixed resins. Results are shown in Figs. 5 and 6. α -Amylase was successfully dialysed by this method (Fig. 7). All the salt was removed and the enzyme rendered inactive, the activity being recoverable by the addition of salt. This method of dialysis is only suitable for very dilute (approx. 0.001M) salt solutions.

SUMMARY

1. The use of ion-exchange resins for dialysis has been examined, and it has been found that proteins can be damaged by contact with the resins.

2. It is still possible to use resins for dialysis if special precautions are taken. A membrane must be used to separate the resins from the solution to be dialysed, and the tendency of the solution to become acid must be prevented by modification of the membrane or by other means. It is then possible to remove ions from enzyme solutions with resins without denaturation.

REFERENCES

- McCready, R. M. & Hassid, W. Z. (1943). *J. Amer. chem. Soc.* **65**, 1154.
 Reid, A. F. & Jones, F. (1949). *Amer. J. clin. Path.* **19**, 10.
 Richardson, R. W. (1949). *Nature, Lond.*, **164**, 916.