REFERENCES

- Archibald, R. M. (1944). *J. biol. Chem.* **156**, 121.
 Bücher, T. & Garbade, K. H. (1952a). *Biochim. biophys. Acta*, **8**, 220.
 Bücher, T. & Garbade, K. H. (1952b). *Biochim. biophys. Acta*, **8**, 222.
 Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, 3rd ed. p. 87. London: Crosby Lockwood.
 Delwiche, C. C., Loomis, W. D. & Stumpf, P. K. (1951). *Arch. Biochem. Biophys.* **33**, 333.
 Doudoroff, M., Barker, H. A. & Hassid, W. Z. (1947). *J. biol. Chem.* **170**, 147.
 Fearon, W. R. (1939). *Biochem. J.* **33**, 902.
 Gale, E. F. (1945). *Biochem. J.* **39**, 46.
 Gornall, A. G. & Hunter, A. (1941). *Biochem. J.* **35**, 650.
 Harden, A. & Young, W. J. (1911). *Proc. Roy. Soc. B*, **83**, 451.
 Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
 Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 461.
 Kawerau, E. (1946). *Sci. Proc. R. Dublin Soc.* **24**, 63.
 Kennedy, E. P. & Barker, H. A. (1951). *J. biol. Chem.* **191**, 419.
 Knivett, V. A. (1952a). *2nd Int. Congr. Biochem., Abstr.*, p. 86.
 Knivett, V. A. (1952b). Ph.D. Thesis, Cambridge University.
 Knivett, V. A. (1954). *Biochem. J.* **56**, 602.
 Korzenovsky, M. & Werkman, C. H. (1953). *Fed. Proc.* **12**, 233.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Needham, D. M. & Pillai, R. K. (1937). *Biochem. J.* **31**, 1837.
 Racker, E. & Krinsky, I. (1952). *J. biol. Chem.* **198**, 731.
 Slade, H. D. (1953). *Arch. Biochem. Biophys.* **42**, 204.
 Stadtman, E. R. & Barker, H. A. (1950). *J. biol. Chem.* **184**, 769.
 Stadtman, E. R., Novelli, G. D. & Lipmann, F. (1951). *J. biol. Chem.* **191**, 365.
 Warburg, O. & Christian, W. (1939). *Biochem. Z.* **303**, 40.

Interaction of Acids of High Molecular Weight with Albumin

By D. HAMER

Cancer Research Laboratories, Department of Pathology, The Medical School, Birmingham 15

(Received 3 June 1953)

Sodium deoxyribonucleate has been shown to prevent the coagulation by heat of dialysed albumin solutions at about pH 5.5 (Carter & Greenstein, 1946; Greenstein & Hoyer, 1950). Evidence of such interactions between nucleic acids and proteins is of considerable interest, since one of the main problems of nucleoprotein structure is the way in which the protein and nucleic acid are bound together. In the experiments described here it is shown that polyacids in general are able to interact with albumin so as to prevent heat coagulation. A short account of this work has already been presented (Hamer, 1953a).

EXPERIMENTAL

Albumin solutions. Bovine plasma albumin (fraction V, Armour and Co.) was used throughout this work. A solution was made up containing approx. 2 g./100 ml. and the pH was adjusted to 6.7–6.8. The solution was then dialysed using cellulose casings (Visking Corp., Chicago) at 2–4° against distilled water. After 4 or 5 days the pH dropped to about 5.5–5.7 and the final protein concentration was then 1.4 g./100 ml., as estimated from the N content.

Other materials. Deoxyribonucleic acids were prepared from isolated calf-thymus nuclei: specimen 1 by saline extraction followed by treatment of the solution with chloroform:butanol mixtures and specimen 2 by saline extractions followed by deproteinization with sodium dodecyl sulphate (Hamer, 1953b). A commercial sample of

yeast ribonucleic acid was used (British Drug Houses Ltd.). Heparin and alginic acid were also obtained from commercial sources. The heparin had an activity of 90 i.u./mg. (Evans, Lescher and Webb Ltd., batch N10090) and a molecular weight of probably 17000 (Jensen, Snellman & Sylven, 1948). The alginic acid had a molecular-weight range of 10000–20000 (specimen HS/LD and details, including the mol. wt. which is based on viscosity determinations, supplied by Alginate Industries Ltd.). Dextran sulphate and dextran were supplied by Dr K. Walton. Both had molecular weights about 8000, as determined by measurements of osmotic pressure and intrinsic viscosity (see Walton, 1952; Ricketts, 1952). Polyglutamic acid was prepared from the capsular substance of *Bacillus subtilis* by the method of Hanby & Rydon (1946), the final product being dialysed and freeze-dried (supplied by F. W. Moore, Bacteriology Department, Birmingham University). The molecular weight of material obtained by this method was reported to be at least 15000.

Aqueous solutions (0.1%) of these compounds were prepared; where necessary to neutralize alginic and nucleic acids a trace of alkali was added, and the solutions were dialysed in the cold to remove salts. It may be noted here that the amounts of these solutions used in the tests described below were so small as to have no effect on the pH of the albumin solutions.

Heat coagulation. 2 ml. of albumin solution with 2 ml. of water (or 2 ml. of the solution under investigation) were heated in a boiling-water bath for 10 min. and chilled in cold water. Under these conditions the albumin coagulates to give a milky-white, colloidal solution which is quite stable

and does not flocculate on standing. Turbidities of the solutions were measured on the Hilger Spekker Absorptiometer using neutral glass filters (H 508) and 2.5 mm. cells. The turbidities of protected solutions were expressed as percentages of the value obtained for an unprotected (0.70%) solution.

Optical rotation. Measurements were made with a Hilger Standard Polarimeter using sodium light and a 1 dm. tube. Tests were carried out on 0.7 and 1.28% (w/v) albumin solutions protected with sufficient dextran sulphate or polyglutamate to prevent any opalescence developing on heating. The rotation of these solutions was determined before and after heating and also after the addition of guanidine hydrochloride to final concentration 3.55M.

Metachromatic tests. Mixtures of the same composition as those used in the coagulation experiments were tested with a 1% solution of toluidine blue.

RESULTS

The results obtained for two different specimens of deoxyribonucleate confirm the findings of Greenstein and co-workers (Fig. 1). Percentage turbidity of the protected solution is plotted against the final concentration of nucleic acid phosphorus and it will be seen that 3–4 $\mu\text{g.}$ are sufficient almost completely to prevent the heat coagulation of the albumin. The weight ratio of albumin to nucleate would be about 200:1 at this point. However, contrary to the results of Carter & Greenstein (1946), it was found that yeast ribonucleate had a comparable effect, inhibiting coagulation to about the same extent as specimen 2 (Fig. 1). This difference may be due to the fact that the experiments of Carter & Greenstein were carried out with liver-tissue extracts and not albumin solutions.

To examine whether the protective effect was only characteristic of polyphosphoric acid groupings, heparin and dextran sulphate were tested with

albumin in the same way. These compounds, which may be considered as polysulphates, behaved in the same way as did the nucleates (Fig. 2). Also, though the molecular weight of heparin is about 17000 and that of dextran sulphate about 8000, weight for weight both compounds had the same effect on the albumin coagulation. Complete protection was given by about 1 part of the polysulphate to 300 parts of albumin. A dextran of corresponding molecular weight had no effect, showing that the presence of the acidic groups was essential for the protection of the albumin.

Finally, several polycarboxylic acids were tested. Fig. 3 shows the results obtained with alginate and polyglutamate. Once more a protective effect was observed. This was not quite so strong as with the

Fig. 2. Effect of heparin and dextran sulphate on the heat coagulation of albumin solutions. Albumin concn., 0.70%. O, heparin; ●, dextran sulphate.

Fig. 1. Effect of deoxyribonucleate specimens on the heat coagulation of albumin solutions. Albumin concn., 0.70%. O, specimen 1; ●, specimen 2.

Fig. 3. Effect of polyglutamate and alginate on the heat coagulation of albumin solutions. Albumin concn., 0.70%. O, alginate; ●, polyglutamate.

poly-sulphates and -phosphates, coagulation being prevented by 1 part of polycarboxylate per 100 parts of albumin. Hyaluronate was also effective. It may be added that in all cases plots of percentage turbidity against the logarithm of the polyacid concentration were linear, except at very high and very low turbidities.

Prevention of the heat coagulation of albumin was only obtained in the absence of salts. As the concentration of salt in the solution was increased, the protective effect was lowered and the turbidity of the solution increased. Fig. 4 illustrates a case where sodium chloride was added in increasing amounts to albumin solutions protected with dextran sulphate. When a point was reached at which the salt concentration (by weight) was about 10 times that of the dextran sulphate, a solid mass of coagulum was formed. Addition of salt to a heated protected solution of albumin does not, however, bring about precipitation or flocculation of the protein.

The following compounds of low molecular weight, used as sodium salts, had no effect at a similar concentration (by weight) and pH: glycerophosphate, guanylate, 2-naphthol-3:6-disulphonate, glucuronate and glutamate.

Considerable evidence was put forward by Greenstein & Hoyer (1950) to show that on heating, the protected albumin was in fact denatured, but that the interaction with the deoxyribonucleate prevented coagulation of the denatured protein. It is probable that a similar process occurs with the other compounds studied here. Some additional evidence has been found for albumin solutions protected with dextran sulphate or polyglutamate, by following the

Fig. 4. Effect of increasing concentration of NaCl on the heat coagulation of albumin solutions protected with dextran sulphate. ○, albumin (7.0 mg./ml.) and dextran sulphate (0.05 mg./ml.); ●, albumin (7.0 mg./ml.) and dextran sulphate (0.10 mg./ml.). || indicates coarse coagulation.

optical rotation. On heating, there was an increase in $[\alpha]_D^{25}$ from -60.4° to -72.9° (independent of the protecting agent and the albumin concentration), showing that some molecular rearrangement had occurred. Jirgensons (1952) has shown that optical-rotation changes are a very sensitive guide to denaturation in albumin and other proteins. Possibly the change occurring in the molecule is more limited than, or of different character from, that caused by guanidine hydrochloride, where extensive denaturation is produced. Addition of guanidine hydrochloride to these heated solutions produced an additional change in $[\alpha]_D^{25}$ to -100.3° . This is comparable with the rotation of an unprotected albumin solution similarly denatured with guanidine hydrochloride, which was found to be -102° .

The polysulphates and polycarboxylates studied gave the characteristic red-purple metachromatic reaction with toluidine blue. In the presence of albumin, both before and after heating, no such colour change was observed, indicating that an interaction exists between the protein and the polyacid.

DISCUSSION

In summarizing the experimental observations given above, it may be said that polyacids of molecular weight from about 8000 upwards are able to interact with serum albumin at pH about 5.5 in salt-free solution so as to prevent the coagulation of the albumin when heated. One part by weight of the polyacid is sufficient to prevent the heat coagulation of 100–300 parts of albumin. If one considers a mechanism of denaturation of the type proposed by Haurowitz (1950), then one could picture that, although intramolecular salt bridges in the albumin molecules are broken down by heating, the interaction already existing between the protein and the acid prevents reorientation of the albumin molecules. By such action, formation of intermolecular salt bridges, and hence coagulation, would be prevented. Recently Cheesman (1953) has found evidence of interaction of deoxyribonucleic and ribonucleic acids with proteins by observing the reorientation of protein dipoles in surface films.

It has not been possible at present to obtain a definite relation between the amount of albumin and the character and amount of polyacid necessary to prevent coagulation. The molecular weight of the protecting agent does not seem important, since similar effects were obtained with compounds varying from dextran sulphate (8000) to deoxyribonucleate (probably $1-2 \times 10^6$). However, heat coagulation is prevented when each molecule is balanced by one to two acid groups of nucleic acid, about two acid groups of dextran sulphate and three to four groups of alginate or polyglutamate. There

may well be some additional steric and ionization effects to be considered which would account for these differences in equivalence of acid groups and albumin molecules.

SUMMARY

1. Polyacids interact with albumin in salt-free solutions to prevent coagulation of the protein by heat. Polyphosphates (nucleic acids), polysulphates (heparin and dextran sulphate) and polycarboxylates (alginic acid and bacterial polyglutamic acid) were all effective. Effects on optical rotation of the albumin were also demonstrated.

2. The protecting agent acts by preventing the coagulation of the albumin, although denaturation occurs to some extent. The molecular size of the protecting agent seems of less importance than the number of acid groups in determining the degree of protection.

This work was carried out in the laboratories of the Birmingham branch of the British Empire Cancer Campaign.

REFERENCES

- Carter, C. E. & Greenstein, J. P. (1946). *J. nat. Cancer Inst.* **7**, 29.
 Cheesman, D. F. (1953). *Biochim. biophys. Acta*, **11**, 439.
 Greenstein, J. P. & Hoyer, M. L. (1950). *J. biol. Chem.* **182**, 457.
 Hamer, D. (1953*a*). *Biochem. J.* **54**, xxx.
 Hamer, D. (1953*b*). *Brit. J. Cancer*, **7**, 151.
 Hanby, W. E. & Rydon, H. N. (1946). *Biochem. J.* **40**, 297.
 Haurowitz, F. (1950). *Chemistry and Biology of Proteins*, p. 131. New York: Academic Press Inc.
 Jensen, R., Snellman, O. & Sylven, B. (1948). *J. biol. Chem.* **174**, 265.
 Jirgensons, B. (1952). *Arch. Biochem. Biophys.* **39**, 26.
 Ricketts, C. R. (1952). *Biochem. J.* **51**, 129.
 Walton, K. (1952). *Brit. J. Pharmacol.* **7**, 370.

Phospholipids

2. ESTIMATION OF AMINO NITROGEN IN INTACT PHOSPHOLIPIDS

BY C. H. LEA AND D. N. RHODES

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

(Received 14 September 1953)

During the course of work on the separation of phospholipids by chromatographic procedures the need became apparent for a rapid micromethod of detecting and estimating amino-containing phospholipids, particularly phosphatidylethanolamine, in the presence of much larger quantities of lecithin (phosphatidylcholine)—if possible without prior hydrolysis of the phospholipid.

The procedure previously employed involves a lengthy hydrolysis with acid or alkali followed by removal of the liberated fatty acids and estimation of amino nitrogen in the water-soluble portion of the hydrolysate, usually by the Van Slyke method. Direct application of the Van Slyke method to the unhydrolysed phospholipid has been attempted but is subject to serious errors and requires a comparatively large quantity of material. Ninhydrin has been used qualitatively for the detection of free amino-containing phospholipids on paper chromatograms by Hecht & Mink (1952), Hack (1953) and others.

It has been suggested (Lea & Rhodes, 1953) that ninhydrin in the buffered methyl cellosolve reagent used by Moore & Stein (1948) for the estimation of

amino acids, and by Levine & Chargaff (1951) for the estimation of nitrogenous constituents in lipid hydrolysates, might be applicable to the determination of unhydrolysed amino-containing phospholipids. The present paper describes this application and records some observations on the behaviour of phospholipids and of simpler fatty esters in the direct Van Slyke determination.

EXPERIMENTAL

Materials

Egg phospholipid. Crude hen's egg phospholipid was prepared by extraction of the freeze-dried yolk with methanol:chloroform (1:1, v/v) followed by evaporation, solution in chloroform, filtration and precipitation several times from ether at 0–3° with acetone (4 vol.). Contaminating, non-phospholipid substances containing amino N (particularly amino acids) were removed by passage in chloroform:ethanol solution through a cellulose column (Lea & Rhodes, 1953).

'Pure' lecithin. Phosphatidylcholine, prepared from egg phospholipid by the chromatographic (alumina column) method of Hanahan, Turner & Jayko (1951), contained no amino N detectable by the Van Slyke method after hydrolysis, and possessed an atomic N:P ratio of 1.01. As shown