soluble collagen increases throughout the experimental period.

5. The highest specific activity is found in neutral-salt-soluble collagen, maximum activity occurring at 10 hr., with a half-life of 18–20 hr. The activity–time curves of acid-soluble collagen and insoluble collagen are similar, maximum activity occurring at 12 and 24 hr. respectively and being very much lower than the activity of neutral-salt-soluble collagen.

6. It is concluded that neutral-salt-soluble collagen is the precursor of collagen fibres and can either be transformed into acid-soluble collagen or add on to pre-existing acid-soluble collagen and insoluble collagen.

7. A water-soluble protein containing hydroxyproline has also been found.

I am indebted to Dr G. Williams for the histological examination of the tissues and to Mr J. P. Bentley for his valuable technical assistance. The carrageenin was the gift of Mr L. Stolloff of Seaweed Chemicals Inc., New Brunswick, to whom I express my thanks.

REFERENCES

- Banfield, W. G. (1955). *J. Geront.* **10**, 13.
- Bowes, J. H., Elliott, R. G. & Moss, J. A. (1953). In *The Nature and Structure of Collagen*, p. 199. London: Butterworth.
- Bowes, J. H., Elliott, R. G. & Moss, J. A. (1955). *Biochem. J.* **61**, 143.
- Bowes, J. H., Elliott, R. G. & Moss, J. A. (1956). *Biochem. J.* **63**, 1 p.
- Bruce, H. M. & Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 501.
- Cameron, G. R. (1952). *The Pathology of the Cell*. Edinburgh: Oliver and Boyd.
- Dunphy, J. E. & Udupa, K. N. (1955). *New Engl. J. Med.* **253**, 847.
- Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
- Gross, J. (1950). *Amer. J. Path.* **26**, 708.
- Gross, J., Highberger, J. H. & Schmitt, F. O. (1955). *Proc. nat. Acad. Sci., Wash.*, **45**, 1.
- Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A. (1954). *Biochem. J.* **56**, 558.
- Henriques, B. O., Henriques, S. B. & Neuberger, A. (1955). *Biochem. J.* **60**, 409.
- Highberger, J. H., Gross, J. & Schmitt, F. O. (1951). *Proc. nat. Acad. Sci., Wash.*, **37**, 286.
- Jackson, D. S. (1953). *Biochem. J.* **54**, 683.
- Jackson, D. S. (1956a). *Biochem. J.* **62**, 25 p.
- Jackson, D. S. (1956b). *Biochem. J.* **64**, 8 p.
- Jackson, D. S. & Fessler, J. H. (1955). *Nature, Lond.*, **176**, 69.
- Jackson, S. F. (1954a). *Nature, Lond.*, **173**, 950.
- Jackson, S. F. (1954b). *Proc. Roy. Soc. B*, **142**, 536.
- Jackson, S. F. & Smith, R. H. (1955). *Symp. Soc. exp. Biol.* no. 9, p. 89.
- Kodicek, E. & Loewi, G. (1955). *Proc. Roy. Soc. B*, **144**, 100.
- Kramer, H. & Little, K. (1953). In *The Nature and Structure of Collagen*, p. 33. London: Butterworth.
- Kröl, S. (1952). *Biochem. J.* **52**, 227.
- Leplat, G. (1933). *C.R. Soc. Biol., Paris*, **112**, 1256.
- Lowry, O. H., Gilligan, D. R. & Katersky, E. M. (1941). *J. biol. Chem.* **139**, 795.
- Nageotte, J. (1927). *C.R. Soc. Biol., Paris*, **96**, 172.
- Neuberger, A., Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **49**, 199.
- Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **284**, 299.
- Orekhovitch, V. N. (1952). *Commun. 2nd Int. Congr. Biochem., Paris*, p. 106.
- Orekhovitch, V. N. (1955). *Commun. 3rd Int. Congr. Biochem., Brussels*, p. 50.
- Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
- Porter, K. R. & Vanamee, P. (1949). *Proc. Soc. exp. Biol., N.Y.*, **77**, 513.
- Robbins, W. C., Watson, R. F., Pappas, G. D. & Porter, K. R. (1955). *J. biophys. biochem. Cytol.* **1**, 381.
- Robertson, van B. & Schwartz, B. (1953). *J. biol. Chem.* **201**, 689.
- Slack, H. G. B. (1957). *Biochem. J.* (in the Press).
- Stearns, M. L. (1940a). *Amer. J. Anat.* **66**, 133.
- Stearns, M. L. (1940b). *Amer. J. Anat.* **67**, 55.
- Tustanovskii, A. A. (1947). *Biochemistry, Leningr.* **12**, 285.
- Williams, G. (1957). *J. Path. Bact.* (in the Press).
- Windrum, E. M., Kent, P. W. & Eastoe, J. E. (1955). *Brit. J. exp. Path.* **36**, 49.
- Wolbach, S. B. & Howe, P. R. (1926). *Arch. Path. (Lab. Med.)*, **1**, 1.
- Zachariades, P. A. (1900). *C.R. Soc. Biol., Paris*, **52**, 182.

The Stabilization of Purified Human Albumin to Heat

BY MARGARET E. MACKAY AND N. H. MARTIN

M.R.C. External Scientific Staff, The Lister Institute of Preventive Medicine, London, S.W. 1, and The Department of Chemical Pathology, St George's Hospital, London, S.W. 1

(Received 25 June 1956)

Scatchard, Gibson, Woodruff, Batchelder & Brown (1944) recorded changes in the turbidity and viscosity caused by heating solutions of purified human albumin at temperatures up to 57°. They found that at pH 6.8 a 25% solution of albumin in 0.3M sodium chloride could be heated at 57° for 10 hr. without demonstrable change in turbidity.

The clinical need for albumin solutions low in salt concentration which could be sterilized by heat led to the studies of Ballou, Boyer, Luck & Luen (1944) on the effect of organic ions on the thermal stability of albumin. They found, using nephelometric methods, that caprylate, phenylacetate and acetyltryptophan improved the thermal stability of

albumin. Since Brand, Kassell & Saidel (1944) had found that human albumin contained only 0.19 % of tryptophan, nutritional considerations influenced the adoption as a standard for the albumin issued for clinical use in the U.S.A. of a 25 % solution of the Harvard Fraction V stabilized at pH 6.8 with 0.04M sodium acetyltryptophan. This solution could be heated for 10 hr. at 60° without alteration in viscosity or turbidity.

Solutions of albumin prepared by the method of Kekwick & Mackay (1954) and sterilized by irradiation with ultraviolet light were found to be satisfactory for transfusion (Martin, 1954). When Murphy (1953) demonstrated that this technique could not be relied on to destroy the serum hepatitis virus, albumin solutions of 6.25 % protein concentration were heated at 58° for 10 hr. instead of being irradiated, a procedure which Gellis *et al.* (1948) claimed would destroy the virus of hepatitis. No gross change in viscosity or in osmotic pressure was observed after the heat treatment and no untoward effects were observed when the heated material was transfused. However, both electrophoretic and ultracentrifugal analysis suggested that some modification of the albumin had taken place and, since no data could be found in the literature, it was decided to examine electrophoretically and in the ultracentrifuge the effect of controlled heating on albumin, and the modification of these effects resulting from the prior addition of amino acids and their acetyl derivatives to the albumin solutions examined.

MATERIALS AND METHODS

Albumin. The albumin used was fraction AP2 (Kekwick & Mackay, 1954), the material normally supplied for intravenous use. One batch (LA 582) was used for all the experiments. It contained 97 % of albumin and 3 % of β -globulin, as estimated by electrophoresis, and moved as a single component in the ultracentrifuge ($S_{20,w}^0 = 4.20$ for a 1 % solution). This material had been dried from the frozen state from aqueous solution and was reconstituted in 0.1M-NaHCO₃ to a protein concentration of 10 % (w/v).

Amino acids. Solutions (0.1M) of glycine, valine, tyrosine, tryptophan and phenylalanine in 0.15M-NaCl, and 0.1M solutions of acetyl glycine, acetylvaline, acetyltyrosine, acetyltryptophan and acetylphenylalanine in 0.15M-NaCl, were used. The purity of each amino acid was checked by chromatography.

Viscosity measurements. These were carried out in an Ostwald viscosimeter at 37°, and the flow time of the heated material was compared with that of the unheated albumin and with water. The flow time of the standard viscosimeter used was 14 sec. for water at 37°.

Electrophoretic measurements. (a) Moving-boundary electrophoresis: samples were dialysed to equilibrium at 2° against sodium phosphate buffer, pH 8.0, *I* 0.2, and diluted with buffer to a concentration of 1.5 % protein. Measurements were made in the Tiselius (1937) apparatus at 0° with

a potential gradient of 6 v/cm. Optical observations by the diagonal-schlieren method (Philpot, 1938) were photographically recorded with monochromatic light (546 m μ). The photograph was projected with an enlargement of eight diameters and tracings were made. The curves were analysed by reflexion across median lines to determine the quantities of the components present. The results are expressed as the percentage of total protein in a mixture attributable to any single component. The analytical values quoted are the mean from a pair of exposures obtained simultaneously from ascending and descending limbs.

(b) Electrophoresis on paper: analysis of samples was carried out in the apparatus described by Franglen, Martin & Treherne (1955) with a protein concentration of 3.4 % in sodium diethylbarbiturate buffer, pH 8.6, *I* 0.06, on Whatman no. 1 paper at a potential difference of 3.5 v/cm. for 16 hr. The proteins were stained with bromophenol blue, and the protein concentrations estimated by the elution technique of Cremer & Tiselius (1950), 0.02N-NaHCO₃ being used in a Unicam spectrophotometer with a 1 mm. cell and a wavelength of 595–600 m μ . (Franglen & Martin, 1954). Results are shown in graphical form and are accurate to ± 4 %. Since the dye binding of the modified albumin might differ from that of the native material, the eluted fluid from four pairs of results was analysed for N content, and the ratios of the two components were compared with those obtained colorimetrically. There was no significant difference in the results obtained by the estimation of N and those obtained colorimetrically.

Ultracentrifuge measurements. Dialysed samples were diluted to give a concentration of 1 % protein so that the solution contained phosphate buffer, pH 8.0, *I* 0.2, in 0.15M-NaCl. The solutions were subjected to 250 000 *g* in the Svedberg oil-turbine ultracentrifuge, a 12 mm. cell being used. The optical recording was the same as that described for electrophoresis.

EXPERIMENTAL AND RESULTS

Effects of time and temperature of heating. Preliminary experiments were carried out on dilute solutions. Albumin solutions [10 % (w/v) protein at pH 6.5] were diluted with 0.15M-NaCl to a protein concentration of 3.4 %, and 6 ml. portions placed in thin-walled tubes closed with rubber stoppers. The tubes were immersed in a thermostat, the temperature of which was controlled to $\pm 0.4^\circ$. Samples of the material were withdrawn at intervals, immediately cooled to 2° and stored at this temperature, together with an unheated sample, until the heating of all samples had been completed. Albumin solutions held at 43°, 51°, 53°, 56°, 58° and 60° for periods up to 24 hr. were examined by electrophoresis on paper and by viscosity measurements, and compared with the unheated sample. The results of the electrophoretic analyses are shown in Fig. 1, in which the percentage of unaltered albumin as judged by the analysis is plotted against the time of heating.

Viscosity measurements on these samples showed a slight but progressive increase in the flow time compared with unheated albumin (19.2–22.0 sec.,

at 58°; 17 sec. when unheated). At protein concentrations above 6.25 % the albumin will gel at 58° in 1 hr. See also Scatchard *et al.* (1944).

Effects of amino acids on the changes produced by heat. Albumin was diluted with 0.15M-NaCl, and then with solutions of the amino acids listed above dissolved in 0.15M-NaCl, so that the final protein concentration was 3.4 % and the amino acid concentration was 20 mg./g. of protein. As the previous experiments had established that modification had occurred after heating the albumin to 58° for 1 hr., these conditions were used in the experiment. The degree of modification was estimated by electrophoresis on paper. None of the amino acids used prevented the modification of albumin except acetyltryptophan and, to a lesser degree, acetylphenylalanine.

Quantities of acetyltryptophan needed to stabilize albumin. Ballou *et al.* (1944) had shown that a 25 % (w/v) solution of albumin in 0.04M acetyltryptophan (40 mg./g. of protein) would withstand a temperature of 60° for 10 hr., and our own preliminary experiments showed that 20 mg./g. would stabilize albumin dissolved in 0.15M-NaCl. The action of a range of concentrations of acetyltryptophan in a more concentrated albumin solution was then tried. To 10 % (w/v) albumin solution, in 0.1M-NaHCO₃, acetyltryptophan was added to a final concentration of 1, 3 or 5 mg./g. of protein. The solutions were heated to 58° for 1 hr. and examined by electrophoresis on paper. This showed that there was gross modification of the albumin by heat at the lower acetyltryptophan concentrations, and only a slight modification at 5 mg./g. of protein. Albumin solutions containing 5, 10 and 20 mg. of acetyltryptophan/g. of protein (10 % albumin in 0.1M-NaHCO₃) were then heated for 6 hr. at 58°, since these conditions approximated to those used in the preparation of albumin for transfusion. The heated albumin was examined by moving-boundary electrophoresis, and in the ultracentrifuge. Samples of heated albumin containing 1, 3 and 5 mg./g. of acetyltryptophan were also examined in the ultracentrifuge. Results of the electrophoretic examinations are shown in Table 1 and indicate that there was some modification at 5 mg./g. of protein, very slight modification at 10 mg./g. of protein and none at 20 mg./g. of protein.

No alteration in the ultracentrifuge analyses was detected at any concentration of amino acid, including those which had shown modification by both of the electrophoretic techniques.

A solution of albumin (5 g. of protein/100 ml. of 0.15M-NaCl) containing 10 mg. of acetyltryptophan/g. of protein was heated at 58° for 24 hr. Samples examined at intervals showed no modification for 7 hr., but showed progressive changes from 7 to 24 hr. (Fig. 2).

The effects of heat on albumin in solution at pH 6.8 and with acetyltryptophan and tryptophan are shown in Fig. 3.

Effect of heat on conjugation of albumin with 1-anilinonaphthalene-8-sulphonic acid. Samples of a 3.4 % solution of albumin in 0.15M-NaCl, pH 6.8, were coupled with 1-anilinonaphthalene-8-sulphonic acid, as described by Weber & Laurence (1954), before and after heating at 58° for 2 hr. with and without acetyltryptophan. The faster-moving

Fig. 1. Effect of heat on a solution of albumin. Protein concentration: 3.4 g./100 ml. Solvent: 0.15M aqueous solution of sodium chloride, pH 6.8. Ordinates represent the percentage of total albumin surviving after the period of heating shown on the abscissa, computed from electrophoretic analysis. $\perp$ Indicates boundary spreading but no clearly defined second component.

Table 1. *Effect of acetyltryptophan on heat stability of albumin*

Solutions of albumin, with the exception of the control, which was unheated, were heated at 58° for 8 hr.

Acetyltryptophan (mg./g. of protein)	Electrophoretic analysis (% total protein)		
	Albumin first component	Albumin second component	Globulin
0	41.5	53.3	1.2
5	84.2	12.2	3.6
5	84.6	12.7	2.7
10	95.5	1.5	3.0
20	97	0.0	3.0
Control	97	0.0	3.0

electrophoretic component regularly produced a fluorescent conjugate similar to that described by Weber & Laurence, whereas the slower component, which appears as the result of controlled heating of albumin in the absence of acetyltryptophan, failed to produce a fluorescent conjugate with the dye.

Fig. 2. Effect of prolonged heat on a solution of albumin with and without acetyltryptophan. Protein concentration: 5.0 g./100 ml. Solvent: 0.15M aqueous solution of sodium chloride, pH 6.8. O, Acetyltryptophan 10 mg./g. of protein; ●, no acetyltryptophan.

Fig. 3. Electrophoretic and ultracentrifugal diagrams showing the effects of heat on purified human albumin. Electrophoresis: 15 ma for 3 hr. in buffer, pH 8.0, I 0.2. Ultracentrifuge: 250 000 g for 1 hr. in buffer, pH 8.0, I 0.15. A, Unheated albumin; B, albumin heated at 58° for 8 hr.; C, albumin heated at 58° for 8 hr. with 20 mg. of acetyltryptophan/g. of albumin; D, albumin heated at 58° for 1 hr. with 20 mg. of tryptophan/g. of albumin.

DISCUSSION

The use of human albumin as a therapeutic agent makes it essential that every precaution should be taken to prevent the possible transmission of virus infection. Since doubts had been cast on the efficiency of ultraviolet light as a practical means of sterilization it was necessary to re-examine the effect of heat on albumin at concentrations likely to be used for transfusion and at temperatures which have some sterilizing effect.

Our earliest investigations indicated that albumin submitted to temperatures of the order of 58° for more than 1 hr. undergoes some modification, as judged by electrophoresis. Practical experience showed that no harmful effects resulted from the transfusion of the material (Kekwick & Mackay, 1954), but it was obvious that a procedure resulting in a loss of at least 30 % of the primary albumin must be wasteful. Moreover, it cannot be assumed that the 'second component', the result of heating, is an adequate substitute for native albumin.

From our experiments it is seen that controlled heat affects albumin in two stages; there is first the reduction in total net charge observed by electrophoretic analysis. This change is associated with a qualitative change as illustrated by the failure of the second component to associate with 1-anilino-naphthalene-8-sulphonic acid to produce a fluorescent compound. These modifications are observed under conditions which do not produce demonstrable alterations in the ultracentrifugal pattern or gross changes in viscosity. Higher temperatures or prolonged heat result in a change in the ultracentrifugal pattern, which, taken in conjunction with the subsequent changes in viscosity and, finally, actual increase in nephelometric reading, suggest molecular aggregation producing a polydisperse system.

The temperature-time curves also show two distinct phases: the first, almost complete in the first hour, associated with the rapid production of component 2, and the second, a slower change, continuing over the next 24 hr.

The presence of acetyltryptophan appears to modify but not inhibit the first molecular reorientation at concentrations equivalent to three molecules of acetyltryptophan/molecule of albumin.

Tryptophan has no comparable effect at any concentration we have investigated. The effect of acetylation of the 2-amino group may alter the capacity of the carboxyl group of tryptophan to combine with the available basic groups of the albumin.

If heating results in cross-linking involving basic groups it would be reasonable to suppose that acetyltryptophan is effective by blocking cross-linkage. We have no direct evidence which would help to identify the precise groups in the albumin molecule which are involved.

SUMMARY

1. Purified human albumin heated in solution in 0.15 M sodium chloride at pH 6.8 develops a second component.

2. At temperatures of 45° and 50° prolonged heating is required to modify albumin, but at temperatures from 56° to 60° the modification is established in 1 hr.

3. In dilute solution slight but progressive increases in viscosity were noted at 58° if heating was prolonged. Solutions of 5–6.25 % could be heated without gross change in viscosity, but above this protein concentration albumin gels.

4. The action of a number of amino acids and acetyl amino acids on the heat stability of albumin was examined, but all were ineffective except acetyltryptophan, and to a lesser degree acetylphenylalanine.

5. The stabilizing effects of different concentrations of acetyltryptophan were estimated. Solutions containing 20 mg. of acetyltryptophan/g. of albumin may be heated for 10 hr. at 60° without modification of the protein.

6. Moving-boundary electrophoresis proved more sensitive in detecting modification than electro-

phoresis on paper, the ultracentrifuge, or changes in viscosity.

We would like to thank Dr J. R. Laurence for a sample of 1-anilinonaphthalene-8-sulphonic acid.

REFERENCES

- Ballou, G. A., Boyer, P. D., Luck, J. M. & Luen, F. G. (1944). *J. clin. Invest.* **23**, 454.
 Brand, E., Kassell, B. & Saidel, L. J. (1944). *J. clin. Invest.* **23**, 437.
 Cremer, H. D. & Tiselius, A. (1950). *Biochem. Z.* **320**, 273.
 Franglen, G. & Martin, N. H. (1954). *Biochem. J.* **57**, 626.
 Franglen, G., Martin, N. H. & Treherne, J. D. (1955). *J. clin. Path.* **8**, 144.
 Gellis, S., Neefe, J., Stokes, J. jun., Strong, L., Janeway, C. & Scatchard, S. (1948). *J. clin. Invest.* **27**, 239.
 Kekwick, R. A. & Mackay, M. E. (1954). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 286.
 Martin, N. H. (1954). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 286, p. 60.
 Murphy, W. P. (1953). *J. Amer. med. Ass.* **152**, 1421.
 Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
 Scatchard, G., Gibson, S. T., Woodruff, L. M., Batchelder, A. C. & Brown, A. (1944). *J. clin. Invest.* **23**, 445.
 Tiselius, A. (1937). *Trans. Faraday Soc.* **33**, 524.
 Weber, G. & Laurence, D. J. R. (1954). *Biochem. J.* **56**, xxxi.

The Speed of Several Cerebral Reactions Involving the Nicotinamide Coenzymes

BY H. McILWAIN AND M. A. TRESIZE

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

(Received 18 June 1956)

A recent survey of respiration in cerebral systems (McIlwain, 1955) showed that although there is qualitative evidence and general expectation that the greater part of cerebral respiration involves nicotinamide coenzymes and cytochromes, known rates of individual reactions oxidizing the reduced coenzymes are too low to account for the oxygen consumption of the brain or of isolated cerebral tissues. Thus respiration of rat or guinea-pig cerebral cortex with glucose and other substrates can reach 120 or 150 μ moles of O_2 /g. of tissue/hr.; but the tissue has been observed to reduce by reduced cozymase only 220 μ moles of cytochrome *c*/g./hr., corresponding to 55 μ moles of oxygen/g./hr.

To bridge this gap we have investigated the rates at which cerebral tissues catalyse a number of relevant reactions. These have included oxidation of reduced cozymase by cytochrome *c* and by air, and

the corresponding reactions of reduced 2'-phosphocozymase. Interconversion of the two coenzymes has been sought, and also optimum conditions for oxidation of their reduced forms by glutathione.

EXPERIMENTAL

Materials

Cozymase (of 90–95 % purity), cytochrome *c* (salt-free preparation of approx. 90 % purity) and adenosine triphosphate were from the Sigma Chemical Co. and from Boehringer, Mannheim. Ethylenediaminetetraacetic acid (EDTA) was from Eastman Organic Chemicals.

Reduced cozymase and 2'-phosphocozymase. Specimens of approx. 90 % purity were obtained from the Sigma Chemical Co. In addition, solutions of the reduced coenzymes were prepared as follows (see Shuster & Kaplan, 1955; Asnis, 1955). Sodium dithionite (15 mg.) in the stopper of an evacuated Thunberg tube was tipped promptly into 20 mg.