SUMMARY

1. Experiments culminating in the crystallization of rennin are described.
2. Evidence that the crystals are pure rennin is presented.
3. Rennin crystals are of very low solubility in water and can be dried without decomposition.

4. Rennin is probably a protease, acting optimally on haemoglobin at pH 3.7.

The author wishes to express his gratitude to Benger's Ltd., and Messrs B. D. Thornley and S. Hilton, without whose aid this work could not have been carried out, to Dr J. G. Davis and Prof. H. D. Kay of this Institute for their kind interest and encouragement, and to Mr N. J. Gruber for photographing the rennin preparations.

REFERENCES

- Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
 Bergmann, M. & Fruton, S. J. (1941). *Advances in Enzymology*, **1**, 63. New York: Interscience Publishers.
 Berridge, N. J. (1943). *Nature, Lond.*, **151**, 473.
 Berridge, N. J., Davis, J. G., Kon, P. M., Kon, S. K. & Spratling, F. R. (1943). *J. Dairy Res.* **13**, 145.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Davis, J. G. & Davies, W. L. (1934). *J. Soc. chem. Ind., Lond.*, **53**, 1083.
 Fomin, D. (1939). *Molochno Maslodelnaya Prom.* **9**, 16.
 Freudenberg, E. & Buchs, S. (1940). *Schweiz. med. Wschr.* **70**, 249.
 Hankinson, C. L. (1943). *J. Dairy Sci.* **26**, 53.
 Hilton, S. (1941). Private communication.
 Kraut, H. & Tria, E. (1937). *Biochem. Z.* **290**, 277.
 Langmuir, I. (1938). *J. chem. Phys.* **6**, 873.
 Lüers, H. & Bader, J. (1927). *Biochem. Z.* **190**, 122.
 McMeekin, T. L. (1939). *J. Amer. chem. Soc.* **61**, 2884.
 Moriyama, H. & Ohasi, S. (1941). *Science (Japan)*, **11**, 336.
 Northrop, J. H. (1939). *Crystalline Enzymes*. New York: Columbia Univ. Press.
 Oppenheimer, C. (1936). *Die Fermente und Ihre Wirkungen*, Supplement, Lieferung 6. The Hague: W. Junk.
 Pirie, N. W. (1940). *Biol. Rev.* **15**, 377.
 Rao, C. N. B., Rao, M. V. L., Ramaswamy, U. S. & Subrahmanyam, V. (1941). *Curr. Sci.* **4**, 215.
 Steinhardt, J. (1938). *Cold Spr. Harb. Symp. Quant. Biol.* **6**, 301.
 Sumner, J. B. (1926). *J. biol. Chem.* **70**, 97.
 Tauber, H. & Kleiner, I. S. (1932). *J. biol. Chem.* **96**, 745.

Physical Properties of Bovine Serum after Heating with Formaldehyde

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(Received 27 February 1945)

Several workers have tried bovine serum as a substitute for human plasma for transfusion, and in most cases serious reactions were observed (Edwards, 1944). Untreated bovine serum is antigenic and contains agglutinins for all groups of human red cells up to a dilution of 1/16. Cohn (1942) found that human and bovine serum albumins have similar crystal form and physical properties. The transfusion of bovine albumin, however, causes violent reactions though all agglutinins have been removed (Edwards, 1944). Any satisfactory substitute for human plasma must be non-toxic, non-antigenic, free from agglutinins and must have a colloid osmotic pressure similar to that of human plasma.

Since bovine serum is a convenient starting material, attempts were made to modify it so as to make it suitable for transfusion; this was done by

heating it with formaldehyde and ammonia. The clinical results obtained with this material have been reported by Edwards (1944),* and an account is now given of its physical properties. It may be recalled that Van der Scheer, Wyckoff & Clarke (1941) found the formation in heated serum of a complex of large molecular weight migrating electrophoretically with a velocity intermediate between those of albumin and β -globulin. Davis, Hollander & Greenstein (1942) showed that the effects of ultra-violet light are similar to those of heating, but more slowly produced. Kleczkowski (1941*a,b,c*) discusses the antigenic properties of complexes formed by heating mixtures of proteins and the effect of heat on antibodies.

* The reporting, in this paper, of the ultracentrifuge results obtained by one of the present authors at Oxford was done without his knowledge or approval.

Fig. 1. Rennin crystals, $\times 300$ (fraction 2).

Fig. 2. Rennin crystals, $\times 300$ (fraction 1).

Fig. 1. Ultracentrifugal experiments. (a) (i) normal serum and (ii) serum heated 80° for 30 min., 1 hr. 30 min. after start of the run. The ordinate magnification is 2.2 times greater in (ii) than in (i). (b) Serum heated for 30 min. at (i) 50°, (ii) 60°, (iii) 65°, (iv) 70°, 1 hr. 20 min. after start of the run. Same ordinate magnification throughout.

Fig. 2. Electrophoretic experiments. (a) Normal serum (1 hr. 25 min. after start of the run). (b) Serum heated at 73° for 10 min. (1 hr. 25 min. after start of the run). (c) Serum heated at 73° for 30 min. (1 hr. 30 min. after start of the run).

EXPERIMENTAL

(1) *Preparation of material.* Bovine serum was obtained by drawing serum off the clot. It was then either passed through a bacterial filter and stored in sterile bottles, or stored frozen at -12° .

Serum coagulates on heating at 70° for 10 min. To prevent this, 0.2% by volume of 40% (w/v) formaldehyde was added before heating, and after mixing, a volume of ammonia (sp. gr. 0.88) equal to that of the formaldehyde was added to convert excess of the latter to hexamethylenetetramine. All the heated sera referred to below were treated in this manner. The heating was performed by placing small vessels containing the serum in a water-bath of the required temperature. The serum reached this temperature in 1 min. (included in the stated times of heating). After the allotted period, the samples were cooled rapidly. In some experiments the serum was diluted before heating.

(2) *Agglutination.* Heating at 73° for 10 min. was found to destroy all the agglutinins. At 65° , on the other hand, Edwards (1943) found that 3 hr. heating was required.

(3) *Osmotic pressure.* Osmotic pressures were measured by the method of Adair (1925) in collodion membranes containing about 2 ml., and dialyzed against physiological saline, the samples of serum being diluted with saline to a protein content of 4%. The results were as follows:

Sample	Osmotic pressure (mm. H_2O)
Untreated serum	190
Serum + formaldehyde and ammonia	190
Heated at 50° for 30 min.	180
Heated at 60° for 30 min.	140
Heated at 65° for 30 min.	120
Heated at 70° for 30 min.	110
Heated at 80° for 30 min.	90

(4) *Ultracentrifuge measurements.* Sedimentation was carried out in the Svedberg oil-turbine ultracentrifuge at a speed of 1000 rev./sec. and observed by the diagonal schlieren method of Philpot (1938). Two sets of measurements were made. In the first, normal bovine serum was compared with serum heated at 80° for 30 min., the samples being diluted with 0.2M-NaCl to give a protein concentration of 1%. The amounts of material appearing in the boundaries were then compared with the total refractive material of the untreated serum.

Sample	s_{20} (uncorrected) $\times 10^{-13}$		Fraction appearing in boundaries	
	Albumin	Globulin	Albumin	Globulin
Untreated serum	3.46	6.21	0.40	0.39
Heated at 80° for 30 min.	4.13	—	0.10	0

In the second set, the samples heated at 50 , 60 , 65 and 70° and used for osmotic measurements were compared; in each case the treated serum was diluted six-fold with 0.2M-NaCl. The amounts of material appearing in the boundaries are expressed as fractions of the total refractive material of the 50° sample, after it had been cleared by centrifuging.

Sample	s_{20} (uncorrected) $\times 10^{-13}$		Fraction appearing in boundaries		Total
	Albumin	Globulin	Albumin	Globulin	
50°	4.14	6.12	0.71	0.23	0.94
60°	4.39	—	—	—	0.93
65°	4.30	—	0.66	—	0.66
70°	4.30	—	0.48	—	0.48

Pl. 2, fig. 1 gives examples of the sedimentation diagrams obtained. The sedimentation constant of the albumin fraction is somewhat increased by the mildest treatment, but undergoes no further change; the sedimentation constant of the globulin component is unchanged. The amounts of both components undergo a progressive decrease as the severity of the treatment is increased; the globulin is more easily affected, not being visible as a separate component after heating above 60° ; the albumin is still visible after heating at 80° , though greatly decreased in amount.

(5) *Electrophoresis.* Samples of untreated serum and serum heated at 73° for 10 and 30 min. were compared in the electrophoresis apparatus described by Tiselius (1937) and the moving boundaries were observed by the method of Svensson (1939, 1940). The samples were dialyzed against phosphate buffer of pH 8.0 and ionic strength 0.1 and diluted with the same buffer to about 2% with respect to protein. Pl. 2, fig. 2 shows the electrophoresis diagrams obtained.

The untreated serum showed the same components as described by Cohn (1941). The heated samples differ from the untreated in showing no resolution into components, though the migrating material is rather heterogeneous.

(6) *Viscosity.* Viscosity measurements were carried out on samples prepared from a stock serum of protein content 8%. The rotating cylinder method described by Robinson (1939) was used. This method, by alteration of shear rate, differentiates between absolute viscosity due to the volume of the particles in solution and structural viscosity due to asymmetry of the particles. Untreated serum showed a slight structural viscosity. Addition of formaldehyde and ammonia, without heating, markedly increased the absolute viscosity. This effect has been noted by Foster & Biguria (1943), who find that it is due to the effect of formaldehyde on the globulins. Subsequent heating caused a loss of the small structural viscosity effect, but a progressive increase of the absolute viscosity.

Sample	Viscosity/viscosity of water (13°)
Untreated serum	2.52-2.88
Serum with formaldehyde and ammonia	3.21-3.32
Heated at 70° for 10 min.	7.35
Heated at 70° for 20 min.	17.3
Heated at 70° for 30 min.	32.1

DISCUSSION

All these results are consistent with a progressive alteration of the serum proteins by heating with formaldehyde and ammonia. There is an increasing heterogeneity with respect to particle size and to electrophoretic mobility; the heterogeneity accounts for the 'disappearance' of the fractions as seen in the sedimentation diagrams. The fall of osmotic pressure shows that the mean particle size increases. The increase of viscosity is, however, out of proportion to the increase in particle size; the absence of structural viscosity in the heated samples shows

that the abnormal rise in viscosity cannot be due to a greatly increased asymmetry of the particles and it must, therefore, be ascribed to an increased solvation of the altered particles. This applies only to that fraction of the protein which does not appear in the sedimentation diagrams, since the sedimentation constants of the recognizable components are little changed.

SUMMARY

1. Bovine serum heated with formaldehyde and ammonia over a range of conditions shows loss of specific properties and a progressive aggregation of its proteins to form a polydisperse system of larger particles.

2. The osmotic pressure, viscosities and electrophoretic and ultracentrifugal characteristics of a range of samples have been measured.

We are indebted to Dr G. Weber for his help and advice with the viscosity measurements.

REFERENCES

- Adair, G. S. (1925). *Proc. roy. Soc. A*, **108**, 627.
 Cohn, E. J. (1941). *Chem. Rev.* **28**, 395.
 Cohn, E. J. (1942). *Trans. Coll. Phys. Philad.* 4th ser. **10**, 149.
 Davis, B. D., Hollander, A. & Greenstein, J. P. (1942). *J. biol. Chem.* **146**, 663.
 Edwards, F. R. (1943). Private communication.
 Edwards, F. R. (1944). *Brit. med. J.* **1**, 73.
 Foster, S. & Biguria, F. (1943). *J. Lab. clin. Med.* **28**, 1634.
 Kleczkowski, A. (1941*a*). *Brit. J. exp. Path.* **22**, 188.
 Kleczkowski, A. (1941*b*). *Brit. J. exp. Path.* **22**, 192.
 Kleczkowski, A. (1941*c*). *Brit. J. exp. Path.* **22**, 208.
 Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
 Robinson, J. R. (1939). *Proc. roy. Soc. A*, **170**, 519.
 Svensson, H. (1939). *Kolloidzshr.* **87**, 181.
 Svensson, H. (1940). *Kolloidzshr.* **90**, 141.
 Tiselius, A. (1937). *Trans. Faraday Soc.* **33**, 524.
 Van der Scheer, J., Wyckoff, R. W. G. & Clarke, F. L. (1941). *J. Immunol.* **40**, 39.

The Nucleoprotein Content of Fibroblasts Growing *in vitro*

2. THE EFFECT OF TISSUE EXTRACTS

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(Received 17 February 1945)

We have already described experiments on the nature of some of the factors which influence the growth of fibroblasts *in vitro* (Davidson & Waymouth, 1943). In these experiments fibroblast cultures were grown in roller tubes according to the technique of Willmer (1942), and the amount of growth was assessed by making chemical estimations of the nucleoprotein phosphorus (NPP) content of the cultures. In this paper the original observations have been continued and extended.

The technique of estimating the growth of tissue cultures by measuring the increase in nucleoprotein phosphorus (NPP) in the growing tissue was introduced by Willmer (1942) and was recommended by

Cunningham & Kirk (1942). Willmer presumed that such an estimation might give a measure of the amount of nuclear material present, but he pointed out that the presence of nucleic acid as a cytoplasmic constituent in certain tissues was a possible drawback to the method. We have recently shown (Davidson & Waymouth, 1944*a, b*) that mammalian and avian tissues contain two types of nucleic acid, deoxyribonucleic acid in the nucleus and ribonucleic acid in the cytoplasm and that in many tissues the ribonucleic acid is the greater in amount. In liver, for example, some 60-80% of the total nucleic acid is present as the cytoplasmic ribonucleic acid (Davidson & Waymouth, 1944*c*). In the rapidly