several types of esterase, with the possible exception of the magnesium-sensitive aromatic esterases. In most cases, the localization of the total esterase activity would be demonstrated, but the use of selective inhibitors should make it possible to study directly the distribution of esterases other than the cholinesterases in tissues. In all cases, however, parallel investigations by biochemical assay of the types and activities of the esterases present in a tissue should be carried out to make possible a full assessment of the histochemical results.

Since indoxyl esters and α -naphthyl acetate are hydrolysed in a similar manner, any difference in histochemical results obtained with these two types of substrate must be associated with the kinetics of the subsequent dye-forming reaction or with the properties of the dyes formed (Holt, 1956).

SUMMARY

1. The rates of hydrolysis of three indoxyl esters (and of α -naphthyl acetate) by the esterases of human red cells and plasma have been measured and compared with those of the corresponding phenyl and choline esters.

2. All the substrates are hydrolysed by both aceto- and butyro-cholinesterase but the non-choline esters are also hydrolysed by an aliphatic and an aromatic esterase of red cells and an aromatic esterase of plasma. The contribution of the cholinesterases towards the total hydrolysis of the indoxyl esters is far greater than that of the other esterases.

3. The indoxyl and α -naphthyl esters are hydrolysed in a similar manner by the esterases, but only slowly, compared with phenyl esters, by the magnesium-sensitive aromatic esterase of plasma.

4. The aromatic esterases of red cells and plasma are not identical.

5. The relative rates of hydrolysis by the three esterases of red cells depend upon the substrate concentration employed.

6. It is concluded that the use of either indoxyl esters or α -naphthyl acetate as substrates in histochemical staining procedures leads to the demonstration of a mixture of esterases.

This work was supported by a grant to the Middlesex Hospital Medical School from the British Empire Cancer Campaign. The author is indebted to Dr F. Hobbiger for valuable advice and assistance throughout this work, and to Dr S. J. Holt for discussions on the histochemical significance of the results.

REFERENCES

- Adams, D. H. (1949). *Biochim. biophys. Acta*, **3**, 1.
 Adams, D. H. & Whittaker, V. P. (1949). *Biochim. biophys. Acta*, **3**, 358.
 Aldridge, W. N. (1953a). *Biochem. J.* **53**, 110.
 Aldridge, W. N. (1953b). *Biochem. J.* **53**, 117.
 Aldridge, W. N. (1953c). *Biochem. J.* **54**, 442.
 Aldridge, W. N. (1954). *Biochem. J.* **57**, 692.
 Augustinsson, K.-B. (1948). *Acta physiol. scand.* **15**, Suppl. 52.
 Augustinsson, K.-B. (1955). *Acta physiol. scand.* **35**, 40.
 Barnett, R. J. & Seligman, A. M. (1951). *Science*, **114**, 579.
 Gomori, G. (1953). *J. Lab. clin. Med.* **42**, 445.
 Hobbiger, F. (1954). *Brit. J. Pharmacol.* **9**, 159.
 Holt, S. J. (1952). *Nature, Lond.*, **169**, 271.
 Holt, S. J. (1954). *Proc. Roy. Soc. B*, **142**, 160.
 Holt, S. J. (1956). *J. Histochem. Cytochem.* **4**, 541.
 Holt, S. J. & Withers, R. F. J. (1952). *Nature, Lond.*, **170**, 1012.
 Mounter, L. A. (1954). *J. biol. Chem.* **209**, 813.
 Mounter, L. A. & Whittaker, V. P. (1953). *Biochem. J.* **54**, 551.
 Myers, D. K. (1952). *Arch. Biochem. Biophys.* **37**, 469.
 Nachlas, M. M. & Seligman, A. M. (1949). *J. biol. Chem.* **181**, 343.
 O'Brien, R. D. (1956). *J. biol. Chem.* **219**, 927.
 Richter, D. & Croft, P. G. (1942). *Biochem. J.* **36**, 746.
 Underhay, E. E., Holt, S. J., Beaufay, H. & Duve, C. de (1956). *J. biophys. biochem. Cytol.* **2**, 635.

Observations on the Presence of Plasma Proteins in Skin and Tendon

By J. H. HUMPHREY, A. NEUBERGER AND D. J. PERKINS*

National Institute for Medical Research, Mill Hill, London, N.W. 7 and Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2

(Received 16 January 1957)

It was reported in an earlier paper (Harkness, Marko, Muir & Neuberger, 1954) that a protein fraction from the skin of rabbits resembled closely serum proteins both in solubility and electrophoretic behaviour. These proteins were, however, not well

characterized and further work was considered desirable. Apart from the intrinsic interest which is attached to the proteins of connective tissue, it was hoped that a more detailed investigation of this fraction might have a bearing on the metabolism of plasma proteins. Studies by various authors, in which labelled amino acids or proteins were used, have produced fairly conclusive evidence that at

* Present address: Department of Chemical Pathology, St George's Hospital Medical School, Hyde Park Corner, London, S.W. 1.

least half, but probably slightly more, of the total plasma protein present in the body is outside the circulation (e.g. Miller *et al.* 1949; Wasserman & Mayerson, 1951; Myant, 1952; Cohen, Holloway, Matthews & McFarlane, 1956). But there is little quantitative information as to where exactly in the body this extravascular plasma protein is, although the results of Yuile, Lucas, Jones, Chapin & Whipple (1953) indicate that relatively large amounts may be found in the skin. This is also indicated by the observations of Gitlin, Landing & Whipple (1953) who applied Coons's fluorescent antibody technique to tissue sections.

The purpose of the experiments to be described was as follows. In the first place it was desired to have an unequivocal identification of the proteins of this fraction. Secondly, we wished to get a reliable estimate of the quantity of the various proteins; and thirdly, we wanted to study the dynamics of the exchange of the plasma protein present in connective tissue with the plasma protein in the plasma. Finally, it was desired to extend the investigation to tissues other than rabbit skin. For preliminary communication see Humphrey, Neuberger & Perkins (1956).

EXPERIMENTAL

Animals

Rabbits. The animals used were adults weighing 2.3–2.7 kg. and fed on diet no. 18 (Bruce & Parkes, 1946), with a supplement of cabbage and hay once a week.

Rats. Adult Albino rats were used weighing approximately 200 g. and were fed on the National Institute stock diet.

Experimental procedure with iodinated proteins

Two or three days before the injection of iodinated proteins until death NaI (100 mg./l.) was added to the drinking water.

Preparation of material for iodination. In each experiment the animal's own serum was used for iodination. For experiments in which albumin was required, the serum was fractionated by the cold methanol procedure (Pillemer & Hutchinson, 1945). Serum (2 vol.) was diluted with 1 vol. of 0.092M-sodium acetate buffer (pH 6.7), and treated with 7 vol. of aqueous methanol (66%, v/v) pre-cooled to -5° . The serum and buffer were cooled to 0° before the addition of the methanol, and 30 min. after the addition the mixture was centrifuged to remove the precipitated globulins. The supernatant containing albumin was dialysed at 0° against two changes of 0.15M-NaCl and five changes of distilled water to remove the methanol. The aqueous albumin solution was freeze-dried and stored in a tightly stoppered bottle in the dark. Albumin or plasma proteins were labelled with ^{131}I according to the method of McFarlane (1956).

Injection of labelled protein. The required dose (25–800 μC) of ^{131}I -labelled albumin or plasma was injected into the marginal ear vein of the rabbit. The washings from the syringe, needle and vessel, which had contained a known

volume of solution, were collected and counted for ^{131}I activity. The dose injected was thus determined by difference.

Blood samples. Immediately before death a sample of blood was obtained. The serum or albumin for ^{131}I assay was prepared as described above. Under the conditions used for iodination approximately 90% of the protein-bound ^{131}I is found in the albumin. The experiments with whole serum, therefore, largely reflect the behaviour of albumin.

Equilibration experiments

Each animal was injected intravenously with labelled or foreign proteins. After the time chosen for equilibration the animal was bled, killed and immediately skinned. The subcutaneous tissue was dissected free of the dermis and hair.

Extraction. The skin and hair were weighed, cut into strips and tightly rolled. The rolls were buried in powdered solid CO_2 until frozen solid. They were then removed, finely sliced and minced with more CO_2 . The fine powder obtained was extracted with 500 ml. of M/15 Na-K phosphate (pH 8.0) for 24 hr. at 4° . The liquid was separated from the skin by filtration through a double thickness of muslin, followed by filtration through Green's no. 508 $\frac{1}{2}$ filter paper. The skin was further extracted with 500 ml. of buffer each day for 2 days.

Albumin preparation. The phosphate extracts were made 26% (w/v) with respect to anhydrous Na_2SO_4 and kept at 35° overnight. In the morning the globulin precipitate was removed by filtration and discarded. Samples of the filtrate were taken, in the appropriate experiments, for ^{131}I counting and Kjeldahl N analysis. When the albumin was required for physical studies, the filtrate was dialysed against distilled water in the cold room until free of sulphate ions, as judged by the addition of 5 ml. of dialysate to 5 ml. of BaCl_2 (2%, w/v). The albumin solution was then freeze-dried and stored.

Radioactive counting techniques

^{131}I . Samples (3 ml.) of the active solutions, or appropriate dilutions thereof, were counted in a well-type scintillation counter.

Counting ^{14}C in solids. The protein solution to be assayed was made 10% (w/v) with respect to trichloroacetic acid and boiled for 10 min. The precipitated protein was centrifuged the following day. The supernatant was discarded and the precipitate was suspended in the following sequence of solvents: aqueous methanol (50%, v/v), absolute methanol and ether. Each time the precipitate was spun down and the supernatant was discarded. The last traces of ether were removed by leaving the sample in the hot room overnight. The dry sample was counted at infinite thickness, 1 cm.² planchets and an end-window Geiger tube being used.

Counting ^{14}C in gas. The dry sample was prepared as above for end-window counting of the solid. The method of gas counting was that of Bradley, Holloway & McFarlane (1954).

Preparation of ^{14}C -labelled proteins for passive transfer

Albumin. [^{14}C]Phenylalanine-labelled rabbit serum albumin was obtained by fractionation of labelled serum with 26% (w/v) Na_2SO_4 . This preparation is described by Humphrey & McFarlane (1954a).

Antipneumococcus antibody. γ -Globulins containing ^{14}C -labelled antibody were obtained by administration of 3 mc of *Chlorella*-protein hydrolysate to a hyperimmunized

rabbit (Dovey, Holloway, Piha, Humphrey & McFarlane, 1954). The globulin contained 70% antibody specifically precipitable with type III pneumococcus polysaccharide. The specific radioactivity of the antibody was 17 500 counts/min./mg. of C as measured by gas counting.

Preparation of other proteins

Endogenously labelled albumin. [α - 14 C]Glycine (350 μ C) was given intravenously to an adult rabbit. After 6 hr. the animal was bled from the ear and skinned in the usual way.

Horse-serum albumin. This protein was obtained from fresh horse serum by the method of Adair & Robinson (1930). The crude albumin fraction was recrystallized twice.

Immunological methods

Anti-rat serum protein rabbit serum. An attempt was made to identify the serum proteins in skin and tendon extracts by serological methods. Rats were chosen, as it is easy to obtain in rabbits good antibodies to rat-serum proteins. The serum was separated and precipitated with alum (Linggood, 1939). A 3-week intravenous course of the alum precipitate was given to two rabbits weighing 2.5 kg. each. After a further week the animals were bled and the sera obtained were tested for precipitins against the rat serum. Skin extracts for testing were prepared in the manner described for rabbits.

Anti-rabbit serum albumin goat serum. A goat was given two subcutaneous injections of normal rabbit serum (total 1 ml.) incorporated in Freund's adjuvant mixture (Freund & McDermott, 1942). Six months later it received five intravenous injections of electrophoretically prepared rabbit albumin (total 25 mg.) in the course of 17 days, and blood was taken 7 days after the last injection. The resulting antibodies were largely against rabbit-serum albumin.

Identification of rat-serum proteins in extracts from skin. The Oakley & Fulthorpe (1953) modification of Oudin's agar diffusion technique for precipitin was used. A number of serial dilutions of the extract and of normal rat serum were layered over the antiserum.

Estimation of horse-serum albumin

Horse-serum albumin was estimated by means of a rabbit antiserum prepared by intravenous injections of alum-precipitated horse-serum albumin (twice recrystallized). A calibration curve was prepared by mixing in small pointed tubes 0.2 ml. amounts of antiserum with graded quantities (0–280 μ g.) of horse-serum albumin dissolved in normal rabbit serum, and making up to 1 ml. with 0.9% NaCl. The mixtures were left for 2 days at 2°, after which the precipitates were spun down, washed 3 times with 0.9% NaCl at 0°, and dissolved in 4 ml. of 0.1 N-NaOH. The values of $E_{1\text{cm}}^{280}$ were plotted against the quantity of horse-serum albumin added. By this means amounts of horse-serum albumin from 20 to 140 μ g. were estimated with not more than 10% variation between duplicates.

The skin extracts, after concentration by pressure dialysis, were tested by adding varying amounts to 0.2 ml. of antiserum as above, and estimating the horse-serum albumin present from the calibration curve. By always testing more than one amount of any sample it was possible to make sure that the quantities of horse-serum albumin were not such as to bring about antigen excess (in which the amount of precipitate diminishes with increasing antigen).

Values for the horse-albumin contents of the skin extracts were, however, less reproducible than those obtained when the horse albumin was dissolved in rabbit serum.

Preparation of ox-tendon extracts

The achilles tendon of an ox was collected from the slaughter house 2 hr. after the death of the animal and placed in solid CO₂. The tendon was dissected from adhering meat and sliced. The slices were mixed with powdered solid CO₂ and minced to a coarse powder. The CO₂ was allowed to evaporate from the powder before extractions in the cold with M/15 Na-K phosphate (pH 8.0) were begun.

Electrophoresis

Moving-boundary electrophoresis. Protein solutions (approx. 1%) containing serum or extracts of skin or tendon were dialysed for 3 days against ($I=0.2$, pH 8.6) veronal buffer (Miller & Golder, 1950). The dialysed solutions were placed in a Perkin-Elmer electrophoresis apparatus and allowed to migrate for up to 4 hr. at 85 v and 17 ma. Schlieren diagrams were obtained and photographed.

Paper electrophoresis. For control purposes horizontal paper electrophoresis, according to the method of Franglen (1953), was used. The buffer used was 0.05 M-Na-K phosphate (pH 8.0) for both 15 hr. runs at 2.5 v/cm. and 7 hr. runs at 5 v/cm. on no. 1 Whatman filter paper.

RESULTS

Equilibration between the circulating plasma protein and the plasma protein in skin and tendon

Intravenous injection of iodine-labelled plasma proteins into rabbits produced a fairly high radioactivity in the proteins of the soluble fraction of the skin. When the radioactivity was related to the nitrogen contents of the extracts and of plasma, it was found that 4 days after injection the specific activity of the soluble skin proteins was 25% of that of plasma, whereas after 8 days this value increased to 50% (Table 1). In the second series of experiments [131 I]albumin was injected and the albumin fraction prepared from the skin extracts. This was done, since albumin is more easily isolated in a purified form from skin extracts than are the globulins. A number of experiments were performed in which the interval between the injection and killing of the animal was varied between 6 hr. and 20 days. The results obtained (Table 2) show that there are wide variations between animals, but it is clear from the values of the apparent plasma volume per 100 g. of skin that it is at least 6 days before the ratio of activity of the serum albumin in the skin to that of the albumin in the plasma reaches a maximum.

The first phosphate extract from skin was always slightly coloured. Attempts were made to measure the extent of contamination of these extracts by blood, by the use of two different methods in the same rabbit. Homologous serum albumin containing 11 μ C of 131 I was injected into a rabbit

Table 1. *Relative activities of the ^{131}I -labelled proteins in the skin extracts and in the circulating plasma 4 and 10 days after intravenous injection of labelled plasma proteins into two different rabbits*

Duration (days)	Sample	^{131}I counts/min./3 ml. sample	N (mg./ml.)	Counts/min./mg. of N	% of plasma activity
4	Serum	46 500	11.4	4080	100
	Extract 1	2 724	2.94	927	23
	Extract 2	1 589	1.53	1040	25
	Extract 3	5 820	3.96	1320	32
10	Serum	92 770	11.3	8210	100
	Extract 1	4 250	1.17	3640	44
	Extract 2	2 410	0.62	3900	47
	Extract 3	1 540	0.39	3920	47
	Extract 4	1 105	0.27	4090	50
	Extract 5	1 220	0.30	4000	48

Table 2. *Effect of variation of the time interval between injection of labelled albumin and killing of the animal on the radioactivity of the skin proteins*

[^{131}I]Albumin was used. The results are expressed in millilitres of serum, i.e. it is arbitrarily assumed that the skin serum albumin and circulating serum albumin have the same specific radioactivity.

Expt. no.	Duration (days)	Wet wt. of skin (g.)	Serum found in skin (ml.)	Vol. of serum/100 g. of skin (ml.)
19	0.25	150	4.1	2.7
20	2	148	12.0	8.1
24	2	148	7.3	4.9
25	2	156	7.0	4.4
23	3	162	10.0	6.2
14	4	192	10.0	5.2
21	4	155	10.4	6.7
11	6	202	23.0	11.4
15	8	150	19.1	12.7
12	12	185	16.0	8.7
16	16	220	25.5	11.6
13	20	172	20.4	11.9

Table 3. *Estimation of blood and intravascular serum present in rabbit skin, dissected 20 min. after intravenous administration of ^{131}I -labelled albumin*

The weight of the skin was 180 g. — indicates negligible.

	Method of estimation	
	^{131}I -labelled serum (ml.)	Cyanhaematin blood (ml.)
Extract 1	1.0	0.9
Extract 2	0.7	0.4
Extract 3	0.5	—
Extract 4	0	—
Total	2.2	1.3
		(= 0.7 ml. of serum)

weighing 2.5 kg. After 14 min. the animal was killed and skinned as described. The skin was extracted and the γ -radiation of each extract was measured and compared with that of the plasma. The haemoglobin content of each extract was also estimated by the cyanhaematin method (King, Gilchrist & Delory, 1944). The results (Table 3) show that the values obtained for the blood content of the skin by the isotope method are about three times

higher than those found by the haemoglobin measurement. The latter is very insensitive with such dilute solutions. The isotopic albumin method, on the other hand, is more accurate, but it measures not only the intravascular albumin but also the albumin which had already diffused into the extravascular compartment between the time of injection and the arrest of circulation. It is likely therefore that the real value of the intravascular-serum content lies somewhere between the figures calculated from the two experiments, and may be between 0.5 and 0.8 ml./100 g. of skin. This is small compared with the amounts of labelled protein found later in the skin (Tables 1 and 2).

It was considered desirable to compare results obtained by iodine labelling with those found by carbon labelling. A mixture of [^{131}I]albumin and [^{14}C]albumin was injected and the animal was killed after 8 days. The agreement between the values found with the two types of label was satisfactory (Table 4).

Further information about exchange of proteins between plasma and skin was obtained by injecting horse-serum albumin and ^{14}C - or ^{131}I -labelled pneumococcus antibodies and using immunological

Table 4. Comparison of the 'apparent' plasma volume of rabbit skins, after intravenous injection of homologous and heterologous serum proteins, at varying times after injection

'Apparent' plasma volume is defined in Table 2. The methods used for measuring the amounts of horse-serum albumin and of the antibodies are described in the text. The values for horse-serum albumin indicate only the order of magnitude. Those for ^{14}C -labelled antibody are only minimum values. - indicates 'not measured'.

Expt. no.	Duration (days)	Horse-serum albumin	Protein injected		^{14}C -Labelled antibody
			^{131}I -Labelled albumin (ml. of serum found in skin)	^{14}C -Labelled albumin	
10	2	(7.0)	15.8	-	-
20	2	(5.2)	12.0	-	-
24	2	(13.0)	7.3	-	-
25	2	(11.2)	7.0	-	-
23	3	(16.0)	10.0	-	-
14	4	(9.0)	10.0	-	8.5
21	4	(2.1)	10.4	-	-
15	8	-	19.1	16.3	6.6
16	16	-	25.3	-	10.5

methods for semi-quantitative measurement of these proteins in the skin. The amount of transfused ^{14}C -labelled antibody present was insufficient for assay by conventional techniques, but could be estimated by specific precipitation in the presence of known amounts of carrier antibody, followed by measurements of the specific radioactivity of the mixture (Humphrey & McFarlane, 1954b). The volumes of the skin extracts were so large that the recovery of antigen-antibody precipitates would have been very difficult; moreover, in some experiments we were interested in obtaining albumin and globulin separately. For these reasons the globulin fraction from the skin extracts was first precipitated by addition of 18% (w/v) Na_2SO_4 . The precipitate which formed overnight was collected, dissolved in buffer and dialysed before assay of its antibody content. By this procedure some loss of γ -globulin inevitably occurred, and our antibody recoveries (Table 4) must therefore be regarded as giving minimum values only. The agreement between the values found in these experiments with those calculated from the ^{131}I figures is not very good.

Preparation of albumin from serum and skin

The low relative specific activity of the albumin extracted from skin, even after some 20 days of equilibration, can be explained by the presence in the skin of an albumin closely resembling, but not identical with serum albumin. To test this possibility a rabbit (Expt. no. 19) was given by vein $350\text{ }\mu\text{C}$ of $[\alpha\text{-}^{14}\text{C}]\text{glycine}$ and $30\text{ }\mu\text{C}$ of ^{131}I -labelled albumin. After 6 hr. the animal was killed and skinned. The ^{14}C and ^{131}I activities of the albumin fractions obtained from the skin and blood were measured.

Serum. Electrophoresis at pH 8.4 in a column of treated cellulose (Porath, 1956) was carried out on 2 ml. of serum. A current of 22 ma was passed for

Fig. 1. Elution diagram of rabbit serum after electrophoresis for 33 hr. in a treated cellulose column. The albumin fraction selected is indicated.

33 hr. The column was then eluted and the main fraction (Fig. 1) was concentrated by pressure dialysis and freeze-dried. Paper electrophoresis showed only albumin to be present. The ^{14}C specific activity as measured by gas counting was 1970 counts/min./mg. of C.

Skin. The first phosphate extract was treated with 26% (w/v) sodium sulphate, filtered, dialysed in the cold against distilled water and freeze-dried. The dry material was redissolved in water and some in-

soluble material was removed in the centrifuge. The supernatant was concentrated by pressure dialysis against water to a small volume (2 ml.). This material was run on the Porath column for 35.5 hr. at 22 mA. Fractions were eluted and taken as shown in Fig. 2. Fraction 1 (23 mg.) had a specific activity of 414 counts/min./mg. of C. Fraction 2 (7 mg.) had a specific activity of 597 counts/min./mg. of C, and fraction 3 (2 mg.) had specific activity of 957 counts/min./mg. of C. The weights do not represent true proportions of the different substituents present, as substantial losses occurred on the column, and in the subsequent pressure dialysis.

Serological examination of fraction 1 from skin extract

The goat antiserum against rabbit albumin which was used had been shown by the agar diffusion technique to contain one major antibody against rabbit albumin and two minor antibodies against globulins. The antiserum was absorbed with a crude rabbit globulin fraction until no further material precipitated. The serum after this treatment contained 1.8 mg. of specific antibody/ml. against rabbit albumin.

Precipitin reactions. Both serum-albumin preparations and the fraction 1 from the skin extract were tested with 1 ml. of antiserum. The skin extract precipitated antibody (see Fig. 3), but its precipitating power was only 87% of that of the serum albumin. One mg. of fraction 2 was added to 5 ml. of antiserum. After 2 days there was no precipitate, although 1 mg. of fraction 1 had precipitated 8.3 mg. (dry wt.) of antibody by this time. After a further 4 days at 0° fraction 2 gave a 5 mg. (dry wt.) of precipitate. To the supernatant was added 0.8 mg. of pure rabbit albumin which gave during the next day a precipitate weighing 5.4 mg. Fraction 2 was not immunologically equivalent to fraction 1, but contained some material capable of reacting with anti-rabbit albumin, as well as material capable of reacting with another antibody present. The mean value of the specific radioactivity of the serum albumin was 2150 counts/min./mg. of C, while that of the skin albumin was 414 counts/min./mg. of C; thus after 6 hr. the skin albumin had reached 19% of the radioactivity of the circulating albumin. The comparable activity of the ^{131}I -labelled albumin from skin was 20% of that in the serum.

Characterization of albumin

Some physical properties of albumin prepared from the skin and serum of one rabbit were measured. The results of the ultraviolet absorption and optical rotation measurements are shown in Table 5. In 3M-aqueous guanidine hydrochloride solution rabbit albumin denatured instantaneously, as shown by the immediate change in optical rotation. Good

Fig. 2. Elution diagram of 'albumin' fraction of rabbit skin extract after electrophoresis for 35.5 hr. in a treated cellulose column. The three fractions examined are indicated.

Fig. 3. Precipitation curve for rabbit-serum albumin and goat antiserum. O, Rabbit-serum albumin; ●, skin-extract albumin (fraction 1).

Table 5. Comparison of the physical properties of rabbit albumin prepared from serum and skin extracts

	Serum	Skin
Optical rotation $[\alpha]_D$		
(a) in M/15 phosphate (pH 8.0)	-52.3°	-52.3°
(b) in 3M-guanidine-HCl	-84.1°	-83.9°
Ultraviolet absorption ($E_{1\text{cm.}}^{1\%}$)		
(a) 290 m μ in 0.1N-NaOH	0.228	0.229
(b) 276 m μ in 0.1N-HC	0.176	0.185

Table 6. *Comparison of the specific radioactivities of the soluble proteins of tendon and skin of a rabbit after administration of [¹³¹I]albumin*

Material	Counts/ min./ml.	N (mg./ml.)	Counts/min./ mg. of N	Total counts in extract	% of serum
Serum at death	46 500	11.4	4080	—	100
Tendon, extract 1	899	0.77	1170	13 490	27
Tendon, extract 2	269	0.18	1490	1 690	36
Tendon, extract 3	89	0.055	1650	1 240	40
Warm NaOH extract	62	8.88	—	—	—
Skin, extract 2	530	0.51	1039	—	25

agreement was obtained for the optical rotation of both specimens in both the denatured and native states. The ultraviolet absorption spectra in alkaline solutions were identical for both specimens. In acid solution the spectra were identical in the range 280–300 $m\mu$, but the skin albumin showed no maximal absorption at 276 $m\mu$ as did the serum albumin. The increase of absorption in the range 280–250 $m\mu$ for skin albumin was ascribed to the presence of a small quantity of material of cellular origin. Paper electrophoresis showed the albumins from the two sources to have identical mobilities. The immunological identity of the two albumins has already been described.

Tendon. An initial experiment was tried on ox achilles tendon (143 g. wet wt.). The minced tendon was extracted with phosphate buffer and 0.35 g. of a fluffy freeze-dried protein was obtained. Moving-boundary electrophoresis showed the presence of albumin and γ -globulin, but the pigmentation made it difficult to detect other components. The agar precipitin technique with a rabbit anti-ox serum showed the presence of all the main serum proteins. The low yield of protein from tendon may reflect the difficulty of maceration and of quantitative extraction.

In order to assess the rate of equilibration of the tendon plasma proteins with the circulating proteins one experiment was performed with ¹³¹I-labelled plasma in a rabbit. The skin and tendon were extracted in the usual way with phosphate buffer (pH 8). The results obtained are shown in Table 6. The specific activities of the extracts are similar for both tendon and skin. The efficiency of extraction is shown by the extremely low count obtained for the complete solution of the remaining tendon in warm sodium hydroxide. The wet weight of the skin of this rabbit was 300 g., yielding 4.9 g. of soluble proteins resembling plasma proteins. Tendon (5 g.) was removed, yielding 0.1 g. of proteins resembling plasma protein.

A similar experiment was made with rats. The skin extracts were tested immunologically against anti-rat serum protein serum as described in the Experimental Section. After one week at 4° there were four major rings of precipitation in the

Table 7. *Plasma-albumin content of rabbit tissues 3 days after the intravenous injection of [¹³¹I]-labelled albumin*

The results refer to the whole animal, which weighed 2.45 kg. 'Apparent' plasma volume is defined in Table 2.

Tissue	'Apparent' plasma vol. (ml.)
Phosphate-saline extract of subcutaneous connective tissue (material A)	> 1.7
Phosphate-saline extracts of remaining animal connective tissue and fat (material B)	> 17
Phosphate extracts of skin	10

clear agar layer. The pattern of rings produced by the extracts and by the whole serum were indistinguishable.

Distribution of serum proteins in tissues other than skin

A more extensive dissection was done in Expt. 23 in an attempt to obtain some quantitative data on the distribution of plasma proteins. The injected labelled proteins were allowed 3 days in which to equilibrate with the extravascular plasma pools. The animal was skinned as described. The subcutaneous material (A), usually scraped off the skin and discarded, was preserved. The fascia, fat, subcutaneous tissue (inseparable from the platysma and latissimus dorsi muscles) (B) from half the animal was carefully dissected out. The two samples were weighed and homogenized. The homogenates were extracted twice with phosphate buffer pH 7.4. The residual material was dissolved in hot 0.1N-NaOH. The extracts and dissolved residues were counted in the scintillation counter. The results are shown in Table 7. Owing to unavoidable mechanical losses in dissection the figures given are minimum values. It can be seen that the subcutaneous connective tissue and fat other than that of skin contain significant quantities of albumin.

DISCUSSION

Nature of the soluble proteins in skin and tendon

The bulk of the proteins extracted from skin by a neutral or slightly alkaline buffer closely resemble plasma proteins. Thus, the electrophoretic pattern shown by this fraction (Harkness *et al.* 1954) is very similar to that obtained with plasma from the same rabbit. Similar findings are now reported for tendon. The close resemblance in electrophoretic behaviour between the soluble proteins of skin and plasma proteins of the rat has also recently been observed by Boas (1955). However, in many of our experiments the electrophoretic diagrams of the skin extracts revealed the presence of significant amounts of proteins other than plasma proteins. Immunological experiments also show that all the main groups of plasma proteins are present in skin and tendon. Boas (1955) deduced from his electrophoretic diagrams that connective tissue contained relatively more albumin and less β - and γ -globulins than plasma.

In the case of albumin the identification of the material obtained from skin with circulating serum albumin is fairly conclusive. The two proteins show almost identical absorption spectra in acid and alkaline solution; their optical rotations in the native state and after denaturation with guanidine are the same and they are both instantly denatured by guanidine. The very high radioactivities of skin extracts after administration of ^{131}I -labelled albumin are further strong evidence. The experiments with horse-serum albumin and with the labelled antibodies have no quantitative significance, but they strongly support the conclusion that both albumins and globulins can penetrate into the skin in large amounts. The quantitative analysis with anti-rabbit serum-albumin antiserum of the fraction obtained by column electrophoresis clearly indicates that a large part of the skin albumin was serologically indistinguishable from serum albumin. There can thus be no doubt that large quantities of all serum proteins are present in skin. The results reported in Table 3 clearly show that these proteins are not derived from the blood left in the skin samples.

But it must also be concluded that the phosphate-soluble fraction of skin contains proteins other than plasma proteins. This is clearly shown by the analysis of the albumin fraction by column electrophoresis and the serological investigations of the fractions thus obtained. The fact that the specific radioactivity of the skin albumin fraction (calculated on a nitrogen basis) never exceeds 60 % of that of the circulating albumin is further evidence for this conclusion. It was found that the glycine isolated from a hydrolysate of the soluble skin protein fraction 7–8 hr. after administration of

$[\alpha\text{-}^{14}\text{C}]$ glycine had a similar radioactivity to the glycine obtained from the circulating plasma proteins (Harkness *et al.* 1954). In the present work it was observed that 6 hr. after injection of labelled glycine the immunologically characterized serum albumin from skin had only 20 % of the activity of the circulating albumin, whereas other albumin fractions from skin had much higher radioactivities. This suggests that the proteins which resemble serum albumin in their general properties, but differ from it serologically, and which have a higher radioactivity within 6 or 8 hr. after injection of $[\alpha\text{-}^{14}\text{C}]$ glycine, are probably made in the skin. But they may be, at least partly, α - or β -globulins. We have no evidence whether they are intra- or extra-cellular in the living animal.

Rate of exchange of albumin between plasma and skin

The data presented in Tables 1 and 2 and elsewhere in this paper indicate that, although there are some variations between animals, the specific activity of the skin albumin is about 20 % of its maximum value relative to that of circulating albumin, in 6 hr., 50 % in about 3 days and 100 % in 6–8 days. But it is not easy to calculate from such figures the rate of replacement of skin albumin by circulating serum albumin. The specific radioactivity of the latter is steadily decreasing in a rather complicated manner owing to the simultaneous operation of mixing and catabolic processes. There is thus no equilibrium point or equilibration period in the sense that the specific activities of the two albumins will remain identical, once they have become equal. The mathematical problems involved have been discussed by Solomon (1953), Henriques, Henriques & Neuberger (1955) and Campbell, Cuthbertson, Matthews & McFarlane (1956). It can be predicted, however, that, provided that serum albumin is neither formed nor broken down in the skin, the specific activity of the skin serum albumin will exceed that of the intravascular albumin, after the activity of the former has reached its maximum. By applying equations given by the authors above to the scanty data available, it can be calculated that the total skin serum albumin is replaced by intravascular albumin in about 40 hr.; in other words, its replacement rate/day is about 60 %. In view of the nature of the data this must be considered a very crude approximation.

Amounts of plasma protein present in rabbit skin

It was found in this and earlier work that repeated extraction of rabbit skin with phosphate yields about 1.6–1.7 g. of soluble proteins/100 g. of skin. But, although a substantial part of this fraction is plasma protein, the exact proportion cannot be accurately assessed. Quantitative

serological data were obtained with one fraction of albumin obtained by column electrophoresis, but owing to mechanical losses we do not know exactly what proportion of the total albumin this fraction represented. It is likely, however, that about 50 % of the albumin behaves immunologically as serum albumin. A similar rough estimate may be obtained from the results of Table 1. Since the maximum activity of the skin albumin occurs earlier than the eighth day, the specific radioactivity of the skin serum albumin after 8 days must be somewhat higher than that of the intravascular albumin, as discussed above. As the specific activity found was 50 % of that of the intravascular albumin, it follows that somewhat less than 50 % of the total skin albumin was serum albumin. From the equations given by Henriques *et al.* (1955), and the data discussed above, a value of about 40–45 % for the content of serum is obtained. Assuming that the globulin fraction also consists of 40 % plasma globulin, it follows that the amount of plasma protein in skin amounts to approx. 0.7 g./100 g. of skin. Assuming that the plasma protein in skin is extracellular and that 60–70 % of the skin water is outside the cells (Manery & Hastings, 1939), it follows that the plasma-protein content of the extracellular water is approximately 1.0–1.5 %. This value is similar to that found for the protein content of lymph other than liver and intestinal lymph (see Yoffey & Courtice, 1956). This is not surprising since it is believed that the lymph resembles in its protein composition that of the extracellular spaces which it drains.

In a preliminary communication Gitlin, Nakasato & Richardson (1955) reported that they obtained evidence that the myoalbumin of muscle is identical with serum albumin. These authors also found that the plasma-protein content of the extracellular space of muscle is 20–25 % of that plasma. This value is similar to that calculated by us for skin.

A rabbit weighing 3 kg. has a plasma volume of about 100 ml. and a total circulating plasma-protein content of 7.0–7.5 g. Assuming its skin to weigh about 300 g. the amount of plasma protein in the skin is approx. 2 g. This means that the skin contains plasma protein in amounts equivalent to 25–30 % of that present in the circulating plasma. The plasma protein of skin, though less than that of muscle, represents a significant fraction of the total extravascular plasma protein of the body.

The plasma-protein content of tendon appears to vary and to be smaller than that of skin. The work of Gitlin *et al.* (1955) indicates that a large part of the extravascular plasma protein is found in muscle. But skin has a higher concentration of plasma protein than muscle and accommodates a relatively large proportion of the total plasma protein of the body.

SUMMARY

1. The presence of plasma proteins in extracts of skin of rat and rabbit and in extracts of tendon of rat, rabbit and ox was demonstrated by electrophoresis and immunological methods.

2. Plasma proteins and serum albumin labelled with ^{131}I were injected into rabbits. Radioactivities of skin extracts or of the albumin fractions of skin were measured at different times after injection and compared with those of corresponding specimens obtained from blood. Similar experiments were also carried out with ^{14}C -labelled albumin, ^{14}C -labelled antibody and horse-serum albumin.

3. The crude albumin from rabbit skin was shown to resemble circulating serum albumin in its electrophoretic behaviour, its ultraviolet spectrum in acid and alkaline solution, its optical rotation, and its instantaneous denaturation with guanidine.

4. Further partition of this fraction by electrophoresis on treated cellulose columns yielded a major fraction which appeared to be identical with serum albumin as judged by its immunological behaviour. But the crude albumin contained at least two other proteins resembling serum albumin but not identical with it.

5. It is concluded that both tendon and skin contain significant quantities of serum proteins in addition to those present within the blood vessels. The amount present in rabbit skin is approx. 0.7 g./100 g. of skin, which is equivalent to 25–30 % of that found in circulating plasma.

6. Appreciable variations between animals and other uncertainties have made it impossible to calculate accurately the rate of exchange between skin albumin and circulating albumin. However, the data suggest that about 60 % of the skin albumin is replaced every day by circulating serum albumin.

The authors wish to thank Dr A. S. McFarlane and Mr R. C. Holloway for the preparation of ^{131}I -labelled proteins and Mr D. Hart for help with freeze-drying.

REFERENCES

- Adair, G. S. & Robinson, M. E. (1930). *Biochem. J.* **24**, 993.
- Boas, N. F. (1955). *Arch. Biochem. Biophys.* **57**, 307.
- Bradley, J. E. S., Holloway, R. C. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 192.
- Bruce, H. M. & Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 501.
- Campbell, R. M., Cuthbertson, D. P., Matthews, C. M. & McFarlane, A. S. (1956). *J. appl. Rad. Isotop.* **1**, 66.
- Cohen, S., Holloway, R. C., Matthews, C. & McFarlane, A. S. (1956). *Biochem. J.* **62**, 143.
- Dovey, A., Holloway, R. C., Piha, R. S., Humphrey, J. H. & McFarlane, A. S. (1954). *Proc. 2nd Radioisotope Conf., Oxford*, p. 337. London: Butterworth.
- Franglen, G. T. (1953). *J. clin. Path.* **6**, 183.

- Freund, J. & McDermott, K. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
- Gitlin, D., Landing, B. H. & Whipple, A. C. (1953). *J. exp. Med.* **97**, 163.
- Gitlin, D., Nakasato, D. & Richardson, W. R. (1955). *J. clin. Invest.* **34**, 935.
- Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A. (1954). *Biochem. J.* **56**, 558.
- Henriques, D. B., Henriques, S. B. & Neuberger, A. (1955). *Biochem. J.* **60**, 409.
- Humphrey, J. H. & McFarlane, A. S. (1954*a*). *Biochem. J.* **57**, 186.
- Humphrey, J. H. & McFarlane, A. S. (1954*b*). *Biochem. J.* **57**, 195.
- Humphrey, J. H., Neuberger, A. & Perkins, D. J. (1956). *Biochem. J.* **64**, 2*r*.
- King, E. J., Gilchrist, M. & Delory, G. E. (1944). *Lancet*, **1**, 239.
- Linggood, F. V. (1939). *Brit. J. exp. Path.* **20**, 502.
- McFarlane, A. S. (1956). *Biochem. J.* **62**, 135.
- Manery, J. F. & Hastings, A. B. (1939). *J. biol. Chem.* **127**, 651.
- Miller, L. L., Bale, W. F., Yuile, C. L., Masters, R. E., Tishkoff, G. H. & Whipple, G. H. (1949). *J. exp. Med.* **90**, 297.
- Miller, G. L. & Golder, R. H. (1950). *Arch. Biochem.* **29**, 420.
- Myant, N. B. (1952). *Clin. Sci.* **11**, 191.
- Oakley, C. L. & Fulthorpe, A. J. (1953). *J. Path. Bact.* **65**, 49.
- Pillemer, L. & Hutchinson, M. C. (1945). *J. biol. Chem.* **158**, 299.
- Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.
- Solomon, A. K. (1953). *Advanc. biol. med. Phys.* **3**, 65.
- Wasserman, K. & Mayerson, H. S. (1951). *Amer. J. Physiol.* **165**, 15.
- Yoffey, J. M. & Courtice, F. C. (1956). *Lymphatics, Lymph and Lymphoid Tissue*. London: Edward Arnold.
- Yuile, C. L., Lucas, F. V., Jones, C. K., Chapin, S. J. & Whipple, G. H. (1953). *J. exp. Med.* **98**, 173.

The Heterogeneity of Bovine β -lactoglobulin

BY A. G. OGSTON AND M. P. TOMBS

Department of Biochemistry, University of Oxford

(Received 4 February 1957)

Ogston & Tilley (1955) reviewed previous work on the heterogeneity of bovine β -lactoglobulins. A number of workers had concluded, on the evidence of electrophoresis, solubility and immunological measurements, that β -lactoglobulin is heterogeneous. Partial fractionation has been reported by Polis, Schmukler, Custer & McMeekin (1950), by Smithies (1954) and by Préaux, Hulsmans & Lontie (1954).

The meaning of much of this work has been made questionable by the discovery by Aschaffenburg & Drewry (1955) that one or both of two distinct β -lactoglobulins may occur in the milk of individual cows, their occurrence being determined genetically. It now appears likely that in all the work done with β -lactoglobulin prepared from pooled milk both β -lactoglobulins were present, and that the reported fractionations may have been of the two from each other. Only Ogston & Tilley (1955), of previous workers, are known to have worked on material prepared from the milk of single cows; they distinguished 'normal' and 'abnormal' types of β -lactoglobulin by the effect of the concentration of the protein on the electrophoretic pattern.

We describe here measurements made on samples of the two β -lactoglobulins, prepared from the milk of single cows, and on mixtures of the two; our object was to find how far previous observations on heterogeneity can be explained by the properties of these two proteins.

EXPERIMENTAL

Nomenclature. The nomenclature of Aschaffenburg & Drewry (1955) is used here: the protein migrating faster by paper electrophoresis at pH 8.6 is called β_1 -lactoglobulin, and the slower β_2 -lactoglobulin. Polis *et al.* (1950) used the terms β_1 - and β_2 -lactoglobulin for subfractions which appear to bear no simple relationship to the fractions as defined by Aschaffenburg & Drewry.

Typing of cows. Samples of milk from identified cows of the herd of the University of Oxford Department of Agriculture were typed by a slight modification of the method of Aschaffenburg (personal communication). Whole milk warmed to 40° was treated with anhydrous Na_2SO_4 (20 g./100 ml.); after being cooled to 25° it was filtered. The filtrate contained only the whey proteins; these were precipitated with $(\text{NH}_4)_2\text{SO}_4$ (20 g./100 ml. of filtrate) and filtered with the aid of Hyflo Supercel (Johns-Manville Co. Ltd., Artillery Row, London, S.W. 1). The mixture of precipitated proteins and Supercel, with a little water to make it fluid, was dialysed against water for 24 hr. The Supercel was then removed by filtration. The type or types of β -lactoglobulin in the filtrate were then found by paper electrophoresis at pH 8.6.

Preparation of lactoglobulins. Milk was obtained in 5 l. batches from individual cows. Each batch was received within 24 hr. of milking and was processed at once. β_1 - and β_2 -Lactoglobulins were prepared from milk identified as containing only β_1 - or β_2 -lactoglobulin by a slight modification of the method of Aschaffenburg & Drewry (1957); the final, concentrated, solution of lactoglobulin was dialysed first against acetate buffer, pH 5.2 (0.12*M*-sodium acetate; 0.04*M*-acetic acid) and then against three 2 l. batches of