4. There was no evidence of pentose-shunt activity from the labelled-carbon dioxide ratios (1·00–1·18) obtained after incubating spermatozoa with D-[6-^{14}C]glucose and with D-[1-^{14}C]glucose.

5. The labelled-carbon dioxide ratios (0·83–1·10) obtained with [1-^{14}C]acetate and with [2-^{14}C]acetate as substrates indicate that ram, bull and dog spermatozoa have negligible synthetic activity and possess only catalytic amounts of C_3 or C_4 intermediates. The corresponding ratio (2·25) for fowl spermatozoa suggests an influx of unlabelled tricarboxylic acid-cycle intermediates.

6. With labelled acetate and glucose it has been possible to obtain an accurate estimate of the endogenous oxidation in spermatozoa in the presence of an exogenous substrate. The endogenous contribution was less than the exogenous for all species except the fowl.

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The Exchange of ^{131}I-Labelled Lipid and ^{131}I-Labelled Protein between Red Cells and Serum

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The exact nature of the red-cell surface has not yet been determined. Observations on the permeability characteristics (Overton, 1895) and on the birefringence of red cells (Schmitt, Bear & Ponder, 1936) have suggested that the surface is lipid in nature. Analysis of the lipid content of red cells has shown that there is sufficient lipid to form a bimolecular layer around the red cell (Gorter & Grendel, 1925). Others have suggested that the red-cell surface is formed of a mosaic of lipid and the red-cell protein, stromatin, and several possible models have been suggested (Nathanson, 1904; Ponder, 1948; Parpart & Ballentine, 1952; Burgen, 1959). Electrophoresis of red cells carried out at varying pH values has suggested that the phosphoric acid groups of kephalin and related lipids

present at the red-cell surface are responsible for the electrophoretic behaviour. More recently, Seaman & Heard (1960) have provided evidence that other anions are also present at the red-cell surface.

The presence of adsorbed proteins at the red-cell surface has also been suggested. Furchgott & Ponder (1940) thought that albumin was adsorbed in a sufficient quantity to produce a layer 50 Å thick, although later this was doubted (Ponder, 1954). Harvey & Danielli (1938) also thought that protein must be adsorbed on the red-cell surface, to account for the very low surface tension that has been found (Norris, 1939). Although the change in electrophoretic mobility of red cells with changes in pH does not resemble that of glass particles coated with plasma proteins, Harvey & Danielli (1938) thought that the electrophoresis data were compatible with the presence of a firmly bound layer of protein not more than 5 Å thick. Stratton & Jones (1955) studied the reaction between normal red cells and antiglobulin serum and concluded that normal red cells are coated with a globulin-like substance. Chaplin & Cassell (1960) produced antibody to human plasma proteins by the injection of eluates made from thoroughly washed human red cells into rabbits.

During investigations on the uptake of ^{131}I-labelled antibody by red cells, Boursnell, Coombs & Rizk (1953) found that there was always an uptake of ^{131}I by the cells that could not be accounted for by the specific adsorption of labelled antibody. They termed this uptake of ^{131}I 'non-specific adsorption'. We thought that further analysis of this non-specific uptake of ^{131}I from ^{131}I-labelled normal serum might give further evidence of the presence of plasma proteins at the red-cell surface. We have found, in fact, that when plasma proteins are labelled with ^{131}I by the usual methods, a small percentage of the ^{131}I also combines with the unsaturated fatty acids of the plasma lipids, and that the greater proportion of the ^{131}I non-specifically adsorbed is due to the rapid exchange of some of these ^{131}I-labelled lipids. The remainder of the ^{131}I present on the red cells represents protein adsorbed at the red-cell surface.

METHODS

Serum and red cells. Both sterile citrated plasma and red cells were obtained from whole human blood supplied by a blood bank and stored at 4° for between 2 and 21 days before use. The plasma was caused to clot by the addition of $CaCl_2$. Compatible red cells and sera were always used.

Serum fractionation. Albumin was obtained after precipitation of the globulins by 2·05 M-$(NH_4)_2SO_4$. α-, β- and γ-Globulin fractions were obtained by starch-block electrophoresis. γ-Globulin (coefficient of sedimentation 7 s) was separated by using a diethylaminoethylcellulose column (Fahey & Horbett, 1959). γ-Globulin was also prepared by using rivanol (6,9-diamino-2-ethoxyacridine) (Hořejší & Smetana, 1956).

Starch-block electrophoresis. This was carried out by the method of Kunkel & Slater (1952) with 3 ml. of serum. The protein was eluted from 1 cm.-width strips of starch and both the protein concentration and ^{131}I content were estimated. Clearly identifiable α- and β-globulin peaks did not always appear; in these cases the eluates from these regions were concentrated and identified further by electrophoresis on cellulose acetate paper, with whole serum as a standard. All electrophoresis investigations were carried out with a barbiturate buffer (pH 8·6, *I* 0·05).

Estimation of protein concentration. This was carried out by the method of Folin & Ciocalteu, as modified by Kunkel & Tiselius (1951), with either albumin or γ-globulin as standard where appropriate.

^{131}I-Labelling of protein solutions. The method used was a modification of that of Boursnell *et al.* (1953). Free iodine was obtained by mixing 0·1 vol. of 25 mM-KI, 0·11 vol. of 5 mM-KIO_3 and 0·1 vol. of 0·1 N-HCl. Of the solution, 0·31 ml. was used for every 40 mg. of protein. Carrier-free ^{131}I (usually 250 μc) was added to the KI solution before the addition of KIO_3 and HCl. The protein solution was buffered to pH 8·0–9·0 with M-glycine–NaOH buffer. Iodination was allowed to proceed for 10 min., then 0·1 ml. of M-KI was added. After a further 10 min. excess of iodine was removed by an anion-exchange column (Veall & Vetter, 1958) and the labelled protein dialysed for 16 hr. against approx. 500 vol. of phosphate–NaCl at pH 7·4 (Na_2HPO_4–NaH_2PO_4 buffer, 4 mM; NaCl, 0·16 M). The number of iodine atoms per molecule of protein varied between 1:1 and 3:1; the final concentration of the labelled serum was usually about 2 g./100 ml. The iodinated protein solution was centrifuged at 6000*g* for 30 min. before use.

Preparation of red cells. Red cells were washed five times, 5 vol. of 0·16 M-NaCl being used for each wash. The upper layer of packed cells (approximately the upper 10%) was removed with each supernatant to remove white cells and platelets. After the final wash, the lower half of the packed-cell column only was used for the experiments.

Suspension of red cells in ^{131}I-labelled serum. ^{131}I-Labelled serum (1 ml.) was added to 3 ml. of a packed-red-cell suspension (haematocrit, 80–95%), left for 20 min. at room temperature and then washed eight times, with 15–20 vol. of phosphate–NaCl (iso-osmotic with serum) for each wash. The exact volume of red cells used was calculated from a microhaematocrit estimation.

Suspension of red cells in serum-protein fractions. A portion (0·5 ml.) of the protein fraction (albumin, α- and β-globulin, 7 s γ-globulin or fibrinogen) was added to 0·5 ml. of the packed-red-cell suspension. In the experiments on the determination of the amount of protein-bound ^{131}I adsorbed from solutions with varying protein concentrations, doubling dilutions of the protein solutions were made. Further treatment was the same as when ^{131}I-labelled serum was used.

Radioactive estimations. ^{131}I was estimated with a well-type scintillation counter, by using a pulse-height analyser with the channel width set at 7·5 v. The ^{131}I-labelled protein solutions usually gave a counting rate between 100 and 500 counts/min./μg. of protein.

Extraction of ^{131}I *with lipid solvents.* Sera, red cells or starch-block residues were extracted with 20 vol. of ether–ethanol (1:3, v/v) and then re-extracted with a further 5 vol. of ether–ethanol; ^{131}I extracted by ether–ethanol has been termed ^{131}I-labelled lipid and that not extracted has been called ^{131}I-labelled protein.

Subfractionation of lipid-solvent-soluble ^{131}I. For these experiments, red cells and sera were extracted with 20 vol. of chloroform–methanol (2:1, v/v) and the extract was washed with 0·2 vol. of water (Folch, Lees & Sloane-Stanley, 1954) to remove any ^{131}I present as inorganic iodide. The washed extract was dried at low temperature, and the lipid redissolved in ether. Subfractionation of the lipids into phospholipid, neutral fat and unesterified fatty acids was carried out by the method of Borgström (1952). The phospholipids were adsorbed from the ether solution on a silicic acid column (2 g., 325 mesh). The eluate from the column was dried and the lipid dissolved in light petroleum (b.p. 80–100°). The unesterified fatty acids were then extracted twice with an equal volume of alkaline 50% ethanol (ethanol–0·1N-KOH, 1:1, v/v). The ^{131}I content of the light petroleum and the alkaline ethanol was estimated. The ^{131}I content remaining in the light petroleum after extraction with alkaline ethanol was taken to represent neutral fat. The phospholipid ^{131}I was taken to be the difference between the total amount of ^{131}I put on the silicic acid column and the eluate obtained from the column.

Filter-paper electrophoresis. Serum (20 μl.) was placed on Whatman filter paper no. 1 and run for 16 hr. at 5 v/cm. with barbiturate buffer, pH 8·6, *I* 0·05. The proteins were stained with Ponceau-S, and the density of the staining was determined with a photoelectric colorimeter, after soaking in oil. ^{131}I content was estimated by cutting the paper into strips 3 mm. wide and counting each strip separately.

Red-cell ghosts. Ghosts were obtained by lysing red cells in 5 vol. of water and flocculating at pH 5·8. They were washed once with water before use.

Digestion of proteins and butanol extraction. γ-Globulin or red-cell ghosts were digested for 16 hr. at 100° in 10 vol. of N-NaOH. The digest was extracted three times with 0·5 vol. of butanol for each extraction. The butanol fractions were combined and the volume was reduced to about 0·5 ml. in air at 40°, and ascending chromatography carried out.

Ascending chromatography. Identification of iodotyrosine was carried out by ascending chromatography on Whatman paper no. 3 in a butanol–water–acetic acid solvent (4:5:3, by vol.). Mono- and di-iodotyrosine solutions were used for reference. The iodotyrosines were identified by spraying first with a solution prepared by mixing 1 vol. of 1% sulphanilic acid in 10% HCl with 1 vol. of 0·12% $NaNO_2$ in water, drying and then spraying with a saturated Na_2CO_3 solution.

Concentrate of protein solutions. Where necessary, protein solutions were concentrated by dialysis in phosphate–NaCl buffer (iso-osmotic with serum) at 12 lb./in.^{2}

^{51}Cr-*Labelled haemoglobin.* [^{51}Cr]Haemoglobin was obtained by labelling red cells with 100 μc of ^{51}Cr by the method of Mollison & Veall (1955). The cells were then lysed and the ghosts removed at pH 5·8. The [^{51}Cr]haemoglobin was then dialysed for 16 hr. against phosphate–NaCl buffer (iso-osmotic with serum) before use.

Distribution of ^{131}I *in packed cells after centrifuging.* Red cells that had taken up ^{131}I-labelled compounds were washed until less than 5% of the ^{131}I present was removed with each wash. A red-cell suspension in 0·16M-NaCl (haematocrit 30–40%) was then made, placed in polythene tubes, centrifuged at 1500*g* for 55 min., frozen, and the column cut into four or five sections with a knife. In one experiment, ^{131}I content was estimated both before and after extraction with ether–ethanol, and in the other three experiments ^{131}I content was measured after extraction. The haemoglobin contents of all the sections were estimated and the ^{131}I contents of the sections corrected for variations in haemoglobin contents. The counting rate of ^{131}I was low and the coefficient of variation of one of the estimates was as high as 20%.

White-cell counts. Estimations of the white-cell content of the red-cell suspensions were made by the conventional counting-chamber technique. The number of white cells in the white-cell-poor suspensions was certainly less than 25/mm.^{3}, but this latter figure has been used for the calculations.

RESULTS

^{131}I-*Labelled serum.* (*a*) Electrophoresis. The extinction of the Ponceau-S stain and the ^{131}I distribution after electrophoresis of ^{131}I-labelled serum on paper are shown in Fig. 1. All the fractions were labelled, the specific activity of the albumin being greater than that of the globulins.

(*b*) ^{131}I extracted by fat solvents. Eight sera were extracted with ether–ethanol. In seven of the eight the amount of ^{131}I extracted was between 0·7 and 1·9% of the total ^{131}I in the sera, and 7·3% was extracted from the eighth serum. The ^{131}I extracted by the fat solvent represents ^{131}I taken up by the unsaturated fatty acids, together with any ^{131}I remaining as [^{131}I]iodide. Subfractionation of lipid-bound ^{131}I into phospholipid, unesterified fatty acid and neutral-fat fractions showed the following distribution (average of two experiments): phospholipid fraction, 8%; unesterified fatty acid fraction, 11%; neutral-fat fraction, 81%.

Uptake of ^{131}I *by red cells suspended in* ^{131}I-*labelled serum.* When 1 ml. of ^{131}I-labelled serum was added to 3 ml. of packed red cells, between

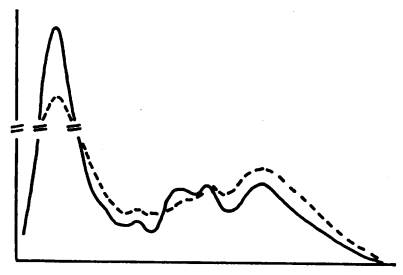

Fig. 1. Paper electrophoresis of iodinated serum. – – –, Extinction of Ponceau-S staining of protein; —, ^{131}I distribution (counts/min.).

0·025 and 0·1 % of the ^{131}I present in the serum usually became firmly bound to the red cells, i.e. it was not removed after eight washes with sodium chloride solution. Between 80 and 95 % of the ^{131}I firmly bound to the red cells could be extracted with lipid solvents.

Subfractionation of lipid-bound ^{131}I taken up by red cells. The lipid ^{131}I taken up by the red cells has been further fractionated in the following way. Red cells (1 ml.) were suspended in 1 ml. of ^{131}I-labelled serum for 20 min. The cells were then separated from the serum and washed eight times with 0·16 M-sodium chloride. The lipid-bound ^{131}I was extracted from both cells and iodinated serum and determinations were made of its distribution in the three fractions: phospholipids, unesterified fatty acid and neutral fat. The total amount of ^{131}I representing phospholipid, unesterified fatty acid and neutral fat in the serum added to the red cells was then calculated. The percentage uptake by the red cells from each of these fractions under these conditions was as follows (values from two experiments): phospholipid fraction, 3·5 and 8·4 %; unesterified fatty acid fraction, 6·0 and 4·2 %; neutral-fat fraction, 0·8 and 0·4 %.

Presence of ^{131}I as iodide in red cells. Although the iodinated serum was passed through an anion-exchange resin and dialysed for 16 hr. against 500 vol. of buffered sodium chloride solution before use, it may still have contained small amounts of ^{131}I present as I^- ion. Although I^- ion is freely diffusible through the cell membrane, and should therefore be removed during the washing of the cells, small amounts were retained by the cells even after eight washes. It is possible to differentiate between [^{131}I]iodide and ^{131}I-labelled lipid by extracting red cells with chloroform–methanol (2:1, v/v). If the chloroform–methanol is shaken with 0·2 vol. of water, and then allowed to separate into two layers, the lipid remains in the lower layer, and 98 % of the [^{131}I]iodide is present in the upper layer. This procedure was carried out on red cells after they had taken up ^{131}I-labelled compounds from iodinated sera and had then been washed eight times. Of the total ^{131}I present in the cells after eight washes, only 10 % was due to [^{131}I]iodide.

Removal of non-specific ^{131}I from red cells. After the red cells had taken up ^{131}I, a considerable proportion of the adsorbed ^{131}I could be removed by suspending the red cells in unlabelled serum. This is illustrated in the following experiments, in which three samples of red cells were used from the same red-cell suspension (Fig. 2). After the red cells had been suspended in ^{131}I labelled serum for 20 min., they were washed eight times with sodium chloride solution; red-cell suspension A was then washed repeatedly with 2 ml. of sodium chloride solution; approximately 3 % of the ^{131}I content was removed with each wash. Red-cell suspension B was washed repeatedly with the same 2 ml. of serum, the serum being removed before estimating the ^{131}I content of the cells and the same sample of serum replaced for each wash. Approximately half the ^{131}I was removed from the cells by the first serum wash, but thereafter the rate of loss was of the same order as the loss occurring from the washes with sodium chloride. Cell suspension C was washed repeatedly with 2 ml. of serum, but with this sample of cells fresh serum was used for each wash. ^{131}I was removed from the cells until a plateau was reached at approximately 10 % of the original amount.

In further experiments, the ^{131}I removed from the cells by the serum washes was further analysed into ^{131}I-labelled lipid and ^{131}I-labelled protein. Fig. 3 shows the amount of radioactivity due to ^{131}I-labelled lipid and ^{131}I-labelled protein left on the red cells after each serum wash. The rate of removal of ^{131}I-labelled lipid was always more rapid and more complete than for the ^{131}I-labelled protein, so that after six washes there was usually only 10–20 % of the ^{131}I-labelled lipid left but 25–80 % of the ^{131}I-labelled protein remained in the red-cell deposit.

Starch-block electrophoresis of serum washes containing exchanged ^{131}I. On seven occasions, ^{131}I-labelled red cells were washed eight times and then suspended in 2 ml. of serum. Some of the ^{131}I-

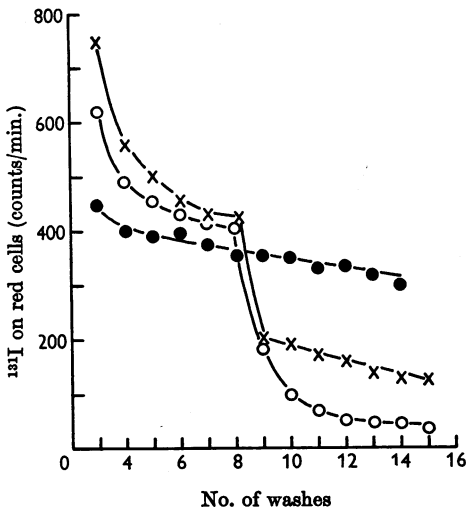

Fig. 2. Removal of ^{131}I attached to red cells by washing. Three samples of red cells were washed eight times with sodium chloride solution. After the eighth wash, sample A (●) was washed with sodium chloride solution; sample B (×) was washed repeatedly with the same 2 ml. of serum; sample C (○) was washed with fresh samples of 2 ml. of serum (for full explanation see text).

labelled lipid and ^{131}I-labelled protein was eluted into the serum, and the serum was then subjected to starch-block electrophoresis. ^{131}I-Labelled lipid and ^{131}I-labelled protein were present in all the fractions (Fig. 4). A high ^{131}I-labelled-lipid content was usually found in the albumin fraction: this probably represents unesterified fatty acids as unesterified fatty acid is mainly bound to albumin (Goodman, 1958). In the β- and γ-globulin regions, considerable amounts of ^{131}I-labelled protein remained attached to the starch granules after thorough washing of the starch. The cause of this is not known. It did not appear to be due to the trapping of particulate matter coated with ^{131}I-labelled protein as it had moved away from the point of application of the serum, and the attachment was still seen after removing particulate matter from the serum either by centrifuging at 1500*g* for 30 min. or by prior filtration through a starch column 5 cm. high.

Adsorption of ^{131}I from solutions of serum-protein fractions. The absorption isotherms of several protein fractions have been determined. The protein fractions were albumin, α- and β-globulin, fibrinogen, 7s γ-globulin and γ-globulin prepared by rivanol fractionation. Examples of the results are shown in Fig. 5. Theoretical Langmuir adsorption isotherms have been calculated and fitted to the experimental results. It can be seen that the adsorption of ^{131}I-labelled protein approximates to that of an adsorption isotherm. All the examples of plasma-protein fractions examined gave similar results. The maximum amount of protein able to be absorbed under the conditions of these experiments would appear to be of the order of 5–15 μg.

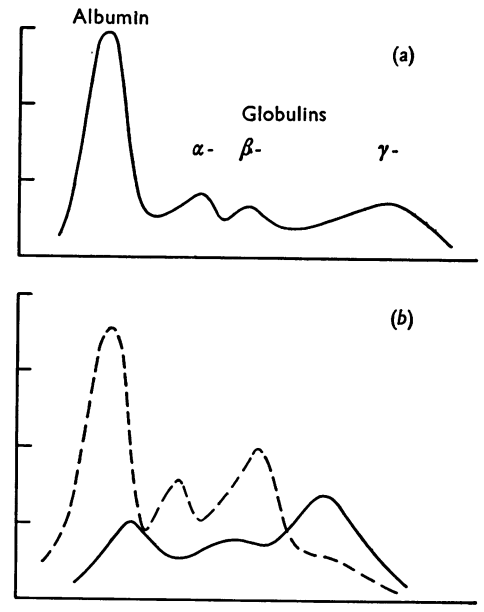

Fig. 4. Starch-block electrophoresis of 2 ml. of serum containing ^{131}I-labelled protein and ^{131}I-labelled lipid derived from the red-cell surface. (*a*) Relative protein concentration. (*b*) Relative ^{131}I distribution in the starch, differentiated into ^{131}I-labelled lipid (– – –) and ^{131}I-labelled protein (—).

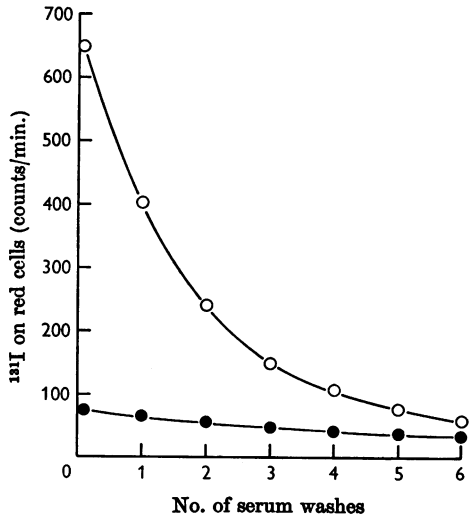

Fig. 3. ^{131}I-Labelled protein (●) and ^{131}I-labelled lipid (○) remaining on 3 ml. of red cells after repeated washing with 2 ml. of serum, fresh serum being used each time. The cells had been washed eight times with sodium chloride solution before the serum washes.

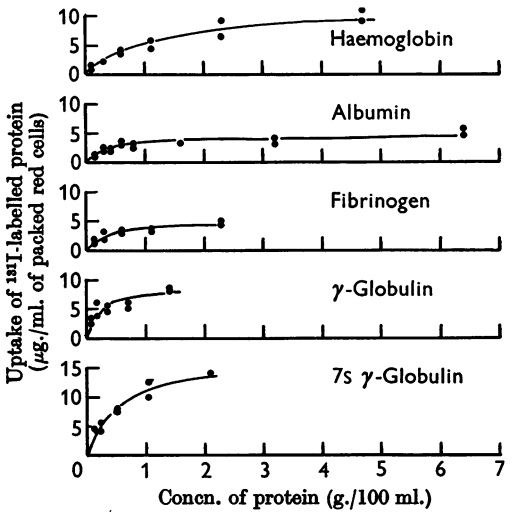

Fig. 5. Uptake of ^{131}I-labelled serum-protein fractions and [^{51}Cr]haemoglobin by red cells from solutions of varying protein concentrations. —, Calculated Langmuir adsorption-isotherms fitted to the data.

of protein/ml. of red cells. The uptake of albumin (five experiments) was consistently lower than the uptake of globulins (six experiments).

The uptake of [^{51}Cr]haemoglobin was also examined. The relationship between haemoglobin uptake and concentration was similar to that of the iodinated serum fractions; the maximum uptake of haemoglobin was of the order of 10 μg./ml. of red cells (Fig. 5).

Inhibition of uptake of ^{131}I-labelled protein by unlabelled protein. In the experiments described so far, the assumption has been made that the adsorption characteristics of the proteins have not been altered by iodination. If the assumption is correct, then the addition of unlabelled protein to iodinated protein should produce a 'homogeneous' population of protein molecules from which the uptake of ^{131}I-labelled protein by red cells should be inversely proportional to the extent of the dilution of the ^{131}I-labelled protein with unlabelled protein. This has been tested by adding increasing amounts of non-iodinated γ-globulin to a constant concentration (0·9 g./100 ml.) of ^{131}I-labelled γ-globulin, and determining the uptake of ^{131}I-labelled γ-globulin by red cells; Fig. 6 shows the ^{131}I-labelled protein uptake by red cells plotted against the extent of the dilution of ^{131}I-labelled γ-globulin with unlabelled γ-globulin. The observed inhibition of ^{131}I-labelled γ-globulin uptake agrees with that expected from the dilution factor, except that the uptake of ^{131}I-labelled γ-globulin in the presence of eight to ten times the amount of unlabelled γ-globulin is higher than expected. In these last-mentioned samples, the radioactivity due to ^{131}I-labelled protein was very low, and it is possible that there was some contamination with ^{131}I-labelled-lipid, owing to failure to obtain complete extraction with ether–ethanol. If the iodination of the γ-globulin had altered its physical characteristics so that it was preferentially adsorbed at the red-cell surface, the uptake of ^{131}I-labelled γ-globulin would not have been inhibited according to the relative concentration of labelled and unlabelled protein. Unlabelled concentrated serum also inhibited the uptake of ^{131}I-labelled γ-globulin in a similar way (Fig. 6).

Effect of temperature on uptake. All the previously described experiments had been carried out with incubation of the cells for 20 min. in the iodinated serum at room temperature. The uptake of ^{131}I-labelled protein and ^{131}I-labelled lipid was determined during incubation of the red cells in a solution of ^{131}I-labelled γ-globulin (2·0 g./100 ml.) at 37° over a 160 min. period (Fig. 7). The uptake of protein progressively increased during this period, so that after 160 min. the amounts of protein adsorbed was 35–50 μg./ml. of cells. Uptake of ^{131}I-labelled lipid also increased from 500 counts/min./ml. of cells at 5 min. to 3000 counts/min./ml. of cells at 160 min.

Investigations on the uptake of ^{131}I by white blood cells. Although efforts were made to remove white blood cells and platelets from red-cell preparations, it is probably not possible to obtain complete removal. Hence ^{131}I uptake by the red-cell suspension may have been due entirely or in part to uptake by contaminating white cells and platelets. The following two experiments were designed to investigate this possibility.

(1) Distribution of ^{131}I in the packed cells after centrifuging. If the presence of ^{131}I were due to uptake by white cells and platelets, then both ^{131}I-labelled protein and ^{131}I-labelled lipid would be found predominantly in the upper layers of the

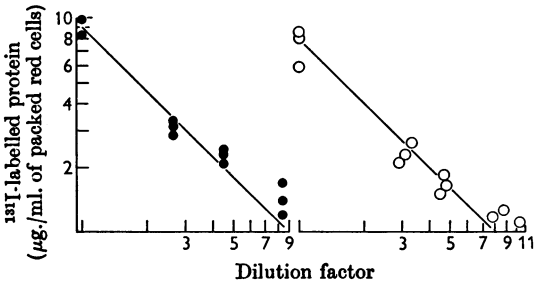

Fig. 6. Inhibition of ^{131}I-labelled protein adsorption on to red cells by the addition of unlabelled protein. ●, ^{131}I-labelled γ-globulin (0·9 g./100 ml.) inhibited by unlabelled γ-globulin; ○, ^{131}I-labelled γ-globulin (1·1 g./100 ml.) inhibited by unlabelled serum. Dilution factor: ratio of total protein concentration to ^{131}I-labelled protein concentration.

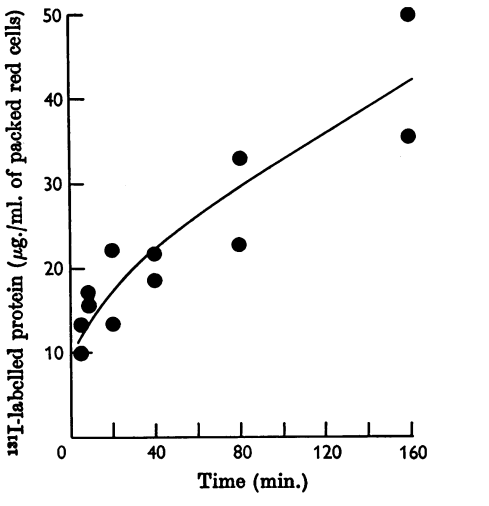

Fig. 7. Uptake of ^{131}I-labelled protein by red cells from ^{131}I-labelled serum during incubation at 37°.

packed-cell column after centrifuging. The results obtained from five experiments of the distribution of ^{131}I in the packed-cell column after centrifuging are shown in Table 1. Although the radioactive concentration was low and the error of the estimations high, there was a fairly even distribution of ^{131}I throughout all the layers, and only in one experiment was the concentration of ^{131}I-labelled protein higher in the top layer. In another experiment a white-cell preparation almost entirely free from red cells was obtained by the method of Nelken, Gilboa-Garber & Gurevitch (1960), and labelled with ^{131}I-labelled serum. No attempt was made to remove platelets from the white cells. A portion ($1{\cdot}4 \times 10^7$) of these ^{131}I-labelled white cells was mixed with 3 ml. of unlabelled red cells and the distribution of ^{131}I in the packed-cell column was determined after centrifuging. Of the ^{131}I, 88% was found in the upper quarter of the column, in the labelled white cells. The remainder of the ^{131}I was evenly distributed in the other layers and was probably due to exchange of ^{131}I from the white cells to the red cells. There is thus a very marked difference between the distribution of ^{131}I in the centrifuged cells after labelling white cells and that seen after labelling red cells.

(2) Comparison between ^{131}I uptake by red-cell suspension with high and low white-cell contents. If the presence of ^{131}I were due to uptake by white cells, then the ^{131}I content should be proportional to the white-cell content. Red-cell suspensions (3 ml.) with high and low white-cell contents were therefore prepared and equal volumes were labelled with ^{131}I-labelled serum from the same batch. After removing all the unadsorbed ^{131}I with sodium chloride solution, the ^{131}I content was estimated and the ^{131}I-labelled lipid was then removed by washing nine times with serum; most of the ^{131}I remaining on the red cells represented ^{131}I-labelled protein (see preceding section). The white-cell-rich suspension contained 800 times the number of white cells in the white-cell-poor suspension (6×10^7 and $7{\cdot}5 \times 10^4$ white cells respectively), but only 4·2 times the amount of ^{131}I-labelled lipid (7000 and 1700 counts/min. respectively) and only 20 times the amount of ^{131}I-labelled protein (4400 and 220 counts/min. respectively). In another experiment, where the cells were washed only with sodium chloride solution, and thus contained ^{131}I-labelled lipids and ^{131}I-labelled protein, there were 300 times as many white cells in the white-cell-rich suspension but only 4·3 times as much ^{131}I.

Both types of experiment show that only a small percentage of the ^{131}I in the red-cell deposit can be due to white-cell contamination. Platelet counts were not made, but if it is assumed that the distribution of platelets is similar to that of the white cells in these experiments, then it may also be concluded that the uptake of ^{131}I was not due to platelet contamination.

Identification of ether–ethanol-insoluble ^{131}I-labelled compound. When proteins are labelled with ^{131}I under the conditions described here, all the ^{131}I is present as [^{131}I]iodotyrosine (Hughes, 1957). The ^{131}I that is not extracted by lipid solvents should therefore represent [^{131}I]iodotyrosine. This has been confirmed by hydrolysing the residue left after ether–ethanol extraction and identifying the ^{131}I as [^{131}I]iodotyrosine. Butanol extracts of alkaline digests of ^{131}I-labelled γ-globulin and of ghosts made from red cells that had been suspended in the ^{131}I-labelled γ-globulin solution were analysed by ascending chromatography on filter paper, mono- and di-iodotyrosine and K^{131}I being used as standards. The iodotyrosine standards did not separate completely and had R_F between 0·5 and 0·7. The greater proportion of the ^{131}I from the red-cell and γ-globulin hydrolysate coincided with the position of the iodotyrosines. A small proportion of ^{131}I travelled with R_F 0·3, coinciding with that of K^{131}I; this ^{131}I was probably split off during hydrolysis. A small amount of a ^{131}I-labelled compound also ran with R_F 0·8, the position of

Table 1. *Distribution of ^{131}I (as total ^{131}I content, or as ^{131}I-labelled protein) in packed-cell columns*

The columns were frozen and cut into four or five sections.

	^{131}I (counts/min.)					
	Total ^{131}I (protein and lipid)		^{131}I-Labelled protein			
Layer						
1 (top)	312*	360	52*	10	10	25
2	295	404	31	9·8	10·3	26
3	260	368	27	9·4	12·2	26
4	244	320	30	10·3	8·8	29
5 (bottom)	274	—	34	10·6	—	—
S.E. of ^{131}I counts (%)	2·5	2·6	16	10	20	10

* Determinations in these columns of figures were made on the same red-cell layers before and after extraction with ether–ethanol.

thyroxine. This thyroxine was probably produced during the hydrolysis of proteins with sodium hydroxide, as thyroxine is formed during the incubation of di-iodotyrosine solutions at alkaline pH (Harington & Pitt-Rivers, 1945).

DISCUSSION

The experiments described here are concerned only with the surface of the cell after the cells have been washed at least six times with sodium chloride solution. Some of the more loosely bound components have therefore probably been removed (Lovelock, 1955). When red cells are suspended in a solution of iodinated plasma proteins, iodinated compounds become attached to the red-cell surface; analysis of these iodinated compounds has shown that there is an exchange of both ^{131}I-labelled lipids and ^{131}I-labelled proteins between serum and the red-cell surface. The exchange of lipids between blood cells and plasma has been suggested previously. Zilversmit, Entenman, Fishler & Chaikoff (1943), Muir, Perrone & Popják (1951) and London & Schwarz (1953) have stated that there is a free exchange of cholesterol between red cells and plasma. Goodman (1958) has shown that there is a rapid exchange of unesterified fatty acids between blood cells and plasma. Hahn & Hevesy (1939) first suggested an exchange of phospholipids between plasma and red cells; Altman, Watman & Salomon (1951) and Lovelock, James & Rowe (1960) have suggested that blood cells synthesize many lipids and that there is an exchange of lipids between the blood cells and the plasma. All these authors, however, used red-cell suspensions which also contained platelets, white cells and reticulocytes, and Buchanan (1960) has shown that almost all the lipid synthesis in these preparations is brought about by the white cells and not the red cells. In the experiments described here particular care was taken to remove the white cells and platelets by centrifuging the cell suspension five times and removing approximately the upper 10% of the packed-cell column on each occasion; then only the lower half of the red-cell column after the final centrifuging was used for the experiments. Moreover, it has been shown that the distribution of the ^{131}I compounds in the packed cells after centrifuging does not correspond to the distribution of white cells or platelets, nor is there any correlation between the white-cell count and the amount of ^{131}I taken up by the cell suspensions. Thus the evidence indicates that it is the red cells themselves that are exchanging lipids. These observations also apply to the adsorption of proteins.

Fractionation of the iodinated lipids attached to the red cells has shown that phospholipids, neutral fat and unesterified fatty acids are all involved in the exchange. The specific activity of the lipids was not determined; so that no quantitative estimate can be made of the extent of the exchange. However, an approximate calculation based on the normal concentration of phospholipids in cells and plasma suggests that under the conditions of these experiments (incubation for 20 min. at room temperature) less than 10% of the phospholipids of the red cells exchanged. In the experiments in which red cells were incubated at 37° in iodinated serum, there was a progressive uptake of ^{131}I-labelled lipids during 160 min., which suggested that *in vivo* there may be considerably greater exchange of red-cell lipid. These findings do not agree with the conclusions of Munn (1958), who found no change in the composition of the unsaturated fatty acids of human red cells after the subjects had taken a meal containing a high proportion of unsaturated fatty acids. He suggested that there was no exchange of fatty acids between plasma and red cells.

When red cells are suspended in iodinated serum, 80–95% of the non-specific uptake of ^{131}I represents ^{131}I-labelled lipid together with a small amount of [^{131}I]iodide. The remainder represents ^{131}I-labelled protein. All the electrophoretically separated fractions of serum proteins were adsorbed as well as a 7S γ-globulin and a [^{51}Cr]haemoglobin. Once the protein has been adsorbed by the red cells, there is only a slow second exchange between the red cells and unlabelled serum. This could indicate that the ^{131}I-labelled protein is diluted on the red-cell surface by unlabelled protein previously adsorbed there, and thus there is more protein on the red-cell surface than is indicated by the uptake of ^{131}-labelled protein at room temperature. The greater uptake of ^{131}I-labelled protein at 37° would be consistent with this view. This hypothesis is also supported by observations in the rat where ^{131}I-labelled protein had been injected intravenously (unpublished work). The uptake of ^{131}I-labelled protein has been found to rise considerably over a period of several hours to values far higher than those obtained at room temperature *in vitro*.

The best evidence that has been produced so far that serum proteins are bound in small quantities to the surface of normal red cells is that of Chaplin & Cassell (1960). They made eluates from thoroughly washed normal red cells and injected them into rabbits. The sera from these rabbits could then be used in the antiglobulin test of Coombs for agglutinating red cells coated with incomplete blood-group antibodies. The rabbit sera contained antibodies against γ-globulin and the complement proteins (now known to be β_1-globulins; Jenkins, Polley & Mollison, 1960). Thus the eluates Chaplin

& Cassell obtained from normal red cells must have contained γ-globulin and β_1-globulin derived from the red-cell surface.

Under the conditions of our experiments, only very small quantities of protein are adsorbed, about 10 μg. of protein/ml. of red cells when the protein concentration of the suspending solution reaches 1–2 g./100 ml. If the average molecular weight of the proteins is taken to be 100 000, then approximately 5000 protein molecules are adsorbed by each red cell. This amount of protein adsorbed by the red cells is of the same order as or even greater than the amount of blood-group antibody taken up during red-cell sensitization (Boursnell *et al.* 1953; P. L. Mollison, personal communication). It is therefore surprising that normal red cells do not give a positive antiglobulin test. However, antiglobulin serum is normally adsorbed with human red cells before use; Stratton & Jones (1955), in fact, have stated that normal red cells are capable of reacting with unadsorbed antiglobulin serum and that this is due to the presence of a globulin-like antigen on the normal red-cell surface.

In the study of the uptake of ^{131}I-labelled antibody by red cells, it has always been found that the non-specific uptake of ^{131}I exceeded that of the ^{131}I-labelled antibody. Boursnell *et al.* (1953) found that the addition of normal serum reduced the uptake of non-specific ^{131}I. This effect can now be attributed to the dilution of the non-specific radioactive protein and lipid. The findings described here show how the non-specific uptake of ^{131}I can be still further reduced. First, the addition of as much non-radioactive protein as possible to the iodinated serum containing antibody will reduce the uptake of non-specific ^{131}I-labelled protein. Secondly, the ^{131}I-labelled lipids and the [^{131}I]iodide can be extracted from the red cells after the cells have been coated with the ^{131}I-labelled antibody. In this way we have been able to reduce the non-specific uptake of ^{131}I to 5 % of that of the ^{131}I-labelled antibody (unpublished work).

SUMMARY

1. The nature of the ^{131}I-labelled compounds taken up by the red cells from iodinated serum proteins has been investigated.

2. During the iodination of serum proteins, the unsaturated fatty acids of the lipoproteins are also labelled with ^{131}I.

3. Under the conditions of these experiments, between 80 and 95 % of the ^{131}I taken up by the red cells from iodinated serum-protein solutions can be extracted with lipid solvents. This ^{131}I mainly represents ^{131}I-labelled lipids that have exchanged between the serum lipoproteins and the red-cell surface. A small amount of the extracted ^{131}I is due to [^{131}I]iodide.

4. Small amounts of iodinated serum proteins are also adsorbed at the red-cell surface. These amounts are dependent on the protein concentration of the suspending solution, and under the conditions of these experiments the maximum uptake of ^{131}I-labelled protein is of the order of 5–15 μg./ml. of red cells.

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The Action of Sulphate and Phosphate Esters of Oestrogens on the Reconstitution of two Pyridoxal 5-Phosphate-Dependent Enzymes

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Mason & Gullekson (1959, 1960) discovered the inhibition of kynurenine aminotransferase (L-kynurenine–2-oxoglutarate aminotransferase, EC 2.6.1.7) and phosphorylase *a* by sulphate esters of stilboestrol [3,4-di-(*p*-hydroxyphenyl)hex-3-ene], oestradiol (oestra-1,3,5-triene-3,17-β-diol) and oestrone (3-hydroxyoestra-1,3,5-trien-17-one), whereas the unconjugated forms were inactive. The authors suggested that the inhibition was due to a competitive mechanism between the sulphate esters and the coenzyme (pyridoxal 5-phosphate).

The kinetics of the inhibition by oestradiol disulphate and stilboestrol disulphate of aspartate aminotransferase (L-aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1) formation from apoenzyme and pyridoxal phosphate was previously studied to contribute further to the understanding of the mechanism of the inhibition (Scardi, Magno & Scarano, 1960).

The purpose of the present work was to investigate whether or not sulphate esterification is necessary to inhibit the reconstitution of pyridoxal phosphate-dependent enzymes, and whether or not there is a specificity of oestrogen esters, both sulphate and phosphate, on the kynurenine aminotransferase. The phosphate esters were chosen because of their solubility and of their possible existence *in vivo*. Aspartate aminotransferase was used because of its role in intermediary metabolism and because its enzymic assay affords a ready method for kinetic analysis of the inhibition.

MATERIALS AND METHODS

Chemicals. Oestradiol disulphate (sodium salt) was prepared by Dr P. de Ruggieri (Ormonoterapia Richter S.p.A., Milan, Italy). The compound examined by paper ionophoresis appeared to be 95% pure (the remaining 5% being monosulphates), though the actual purity might be higher because hydrolysis during ionophoresis cannot be excluded. Oestradiol diphosphate, oestradiol 3-phosphate, oestradiol 17-phosphate and stilboestrol diphosphate were given by AB Leo (Halsingborg, Sweden); the preparation and purity of these compounds have been reported by Fernö, Fex, Högberg, Linderot, Veige & Diczfalusy (1958) and Diczfalusy, Fernö, Fex, Högberg & Kneip (1959) respectively. Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) sulphate as described by D'Alò (1956) was given by Recordati S.p.A. (Milan, Italy). Pyridoxal 5-phosphate, aspartic acid and α-oxoglutaric acid were products of Hoffmann–LaRoche (Basle, Switzerland), and kynurenine sulphate was obtained from California Corp. for Biochemical Research, Los Angeles, U.S.A.

Enzymes and activity determinations. Kynurenine aminotransferase from rat kidney was prepared and assayed as described by Mason (1957). Aspartate aminotransferase was prepared from pig heart by the method of O'Kane & Gunsalus (1947) and freed from pyridoxal phosphate as reported by Scardi *et al.* (1960). The activity was measured spectrophotometrically, according to Cammarata & Cohen (1951), with a Beckman spectrophotometer model DU equipped with thermal spacers and a circulating constant-temperature bath. Protein concentration was calculated from the extinctions at 280 and 260 mμ, according to Kalckar (1947).

The experiments with aspartate aminotransferase were carried out by preincubating apoenzyme with different concentrations of inhibitors and pyridoxal phosphate at