The Effect of Chemical Treatments of Albumin and Orosomucoid on Rate of Clearance from the Rat Bloodstream and Rate of Pinocytic Capture by Rat Yolk Sac Cultured *in vitro*

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Portions of a ^{125}I -iodinated bovine serum albumin preparation were exposed to freezing, acetic acid (pH 3.5, 3.0 or 2.5), urea or formaldehyde, and the effect of these treatments on the rates of pinocytic uptake by yolk sacs from 17.5-day-pregnant rats cultured *in vitro* and of clearance from the rat bloodstream were studied. Uptake of albumin by the yolk sac was followed by rapid release of ^{125}I -iodo-L-tyrosine into the culture medium. Similarly clearance of albumin *in vivo* was accompanied by the appearance of trichloroacetic acid-soluble radioactivity in the bloodstream. In both systems the rates of uptake of modified albumin preparations formed a series: formaldehyde or urea > acetic acid > freezing. The increased rates of uptake of modified albumin preparations could not be ascribed to the formation of aggregates nor, in the yolk-sac system, to an increase in the rate of pinosome formation. It is concluded that the various treatments to which the albumin was subjected increase to varying degrees the affinity of the albumin molecule for binding sites on that region of the plasma membrane from which pinocytic vesicles are formed. Some comparable experiments with native and desialylated human orosomucoid indicate that the rat yolk-sac epithelial cells do not possess the recognition system for uptake of asialoglycoproteins that exists on the surface of hepatic parenchymal cells.

Proteins vary widely in the rate at which they are cleared from the mammalian circulation (see review by Bocci, 1970). No full rationale for the observed differences is available, but in general denatured, modified or heterologous proteins are cleared more rapidly than the equivalent native homologous species. The reticuloendothelial system, particularly the Kupffer cells of the liver, is usually found to be responsible for removal of proteins (and other macromolecules) from the bloodstream.

There is good evidence that protein uptake from the bloodstream occurs by endocytosis, usually followed by intralysosomal proteolysis (Gordon, 1973). The question then arises as to how endocytosis can display sufficient specificity to account for the observed differences in uptake rates. The most plausible explanation is that ingestion of rapidly cleared molecules involves interaction with binding sites on that region of the plasma membrane from which the endocytic vesicles are formed. A mechanism of this type has been shown to operate in the clearance of human glycoproteins from the circulation of the rat (for review, see Ashwell & Morell, 1974), where, except with transferrin, removal of terminal sialic acid residues from the carbohydrate

moieties results in very large increases in the rates of uptake by the liver. This effect was attributed to the exposure of the penultimate galactose residue, since oxidation or removal of this residue restored the clearance rate to a value similar to that for the native glycoprotein. These and other results indicated that there is a positive recognition between the intact terminal galactose residues of the circulating protein and receptor sites on the liver cells, and identified the liver cells involved in uptake of desialylated glycoproteins as parenchymal, not Kupffer, cells.

Although galactose residues may constitute one important recognition signal between circulating protein and the plasma membrane of endocytic cells, other recognition signals must exist, if only for proteins with no carbohydrate moiety. At present no investigation of comparable depth has been made with such proteins, although Normann (1973, 1974a,b) has demonstrated competition during simultaneous clearance of different labelled particles, including aggregated albumin, from the bloodstream of rats and concluded that competition arose through the particles having differing affinities for binding sites on the surface of the Kupffer cells. As indicated above, many studies have been undertaken on rates of clearance of proteins, but these have frequently been made with protein preparations of undeter-

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mined physicochemical characteristics, and are thus difficult to interpret in terms of mechanism. The results of one investigation may relate to a given protein in its monomeric form, and those of another may relate to it in some ill-defined aggregated form. Meaningful comparison of such results is impossible, since two such preparations of the same protein may each be taken up preferentially by a different cell type. Such studies are often further confused by a failure to appreciate that, after its clearance from the circulation, a protein may be digested extremely rapidly and low-molecular-weight digestion products released back into the bloodstream in quantity. Failure to differentiate intact protein from hydrolysis products leads to the rate of clearance of the protein being underestimated. Although confusion from these two sources is preventable by better experimental design, certain complexities are inherent in studies using whole animals. Identification of the relative importance of different anatomical sites of uptake is difficult, since the quantity of a labelled protein found in a tissue does not represent the amount cleared by the tissue, but only the difference between the quantity ingested and the quantity digested by and then released from the tissue. Since the digestion products are released into a common pool, it is impossible to determine the amount of digestion products that originates from a given tissue and thus correct the observed tissue content for the effects of digestion. Even in studies with perfused organs the differential blood supply to individual cells precludes quantitative inferences about the endocytic potential of a particular type of cell being made from the rate of the overall clearance process. It is thus more likely that mechanisms of uptake will emerge from studies with cells cultured *in vitro*.

Williams *et al.* (1975a,b) described an organ-culture system, using yolk sacs from 17.5-day-pregnant rats, in which both ^{125}I -labelled poly(vinylpyrrolidone) and ^{125}I -labelled bovine serum albumin were shown to be ingested at characteristically constant and reproducible rates. Uptake of ^{125}I -labelled poly(vinylpyrrolidone) resulted in a progressive accumulation of radioactivity by the tissue. Uptake of ^{125}I -labelled albumin was followed by extensive proteolysis, and the radioactivity found in the tissue reached a constant value when the rate of production (and release) of labelled digestion products equalled the rate of uptake of the labelled protein. The ^{125}I -labelled albumin was ingested at a markedly higher rate than was ^{125}I -labelled poly(vinylpyrrolidone); this was ascribed to albumin adsorbing to the plasma membrane of the yolk-sac epithelial cells and not to its increasing the rate of formation of pinocytotic vesicles. It was also found that several different batches of ^{125}I -labelled albumin each exhibited a reproducible but different

rate of uptake. It was suggested that such differences might reflect differing degrees of denaturation of the individual preparations.

In the experiments reported here, portions of a single preparation of ^{125}I -labelled bovine serum albumin, a protein with no carbohydrate moiety, were exposed to different chemical agents, and the effects of such treatments, both on the rate of clearance from the bloodstream of the rat *in vivo* and on the rate of ingestion by rat yolk sac *in vitro*, were examined. In addition, the ^{125}I -labelled glycoproteins orosomucoid and asialo-orosomucoid were studied in both systems. A preliminary report of a part of this work has been made (Moore *et al.*, 1974); some of the results have also been discussed elsewhere in relation to intracellular protein turnover (Lloyd, 1976) and to the transmission of immunoglobulins across the rodent and lagomorph yolk sac (Lloyd *et al.*, 1975).

Materials and Methods

Chemicals

[^{125}I]Iodide (preparation IMS.4) and ^{125}I -labelled poly(vinylpyrrolidone) (preparation IM.33P) were from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (preparation 0142t) was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Tissue-culture medium 199 and inactivated calf serum (preparations TC 20 and CS 07 respectively) were from Wellcome Reagents, Beckenham, Kent, U.K. Samples of human orosomucoid and asialo-orosomucoid were provided by Dr. G. Ashwell, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD 20014, U.S.A.

Preparation and treatment of ^{125}I -labelled proteins

Bovine serum albumin. Bovine serum albumin (20 mg) was dissolved in 9.5 ml of 0.05 M- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, pH 8.0, the solution cooled to 0°C and Na^{125}I (2 mCi in 0.5 ml) added. After stirring for 2 min, chloramine-T (4 mg in 5 ml of water) was added and the solution stirred for a further 8 min, when iodination was stopped by the addition of sodium metabisulphite (4 mg in 5 ml of water). KI (33 mg) was added to assist displacement of unchanged [^{125}I]iodide from albumin during the subsequent dialysis. One portion of the preparation, containing 1 mg of protein/ml, was dialysed at 4°C in Visking tubing for 48 h against three changes (5 l each) of 1% (w/v) NaCl. The major part was stored at -20°C, but a small amount was stored at 4°C and used within 1 week of preparation. Further portions were titrated with acetic acid to $\text{pH} 3.5 \pm 0.1$, $\text{pH} 3.0 \pm 0.1$ or $\text{pH} 2.5 \pm 0.1$ and kept at 37°C for 1, 2 and 2 h respectively, then dialysed as described above.

Other portions were treated at 25°C with formaldehyde or urea by mixing a portion of the labelled albumin solution with an equal volume of either formaldehyde solution (10%, w/v, in 0.5M-NaHCO₃ buffer, pH 10) or of aq. 8M-urea solution (pH 5.5); a further portion of the solution was mixed with an equal volume of 0.5M-NaHCO₃ buffer, pH 10, alone. After 72h these solutions of labelled albumin were dialysed at 4°C against three changes (5l) of 1% (w/v) NaCl for 48h, then stored at -20°C.

All of the above preparations were shown to contain less than 1% of trichloroacetic acid-soluble radioactivity when the protein was precipitated by addition of 0.5 vol. of 20% (w/v) trichloroacetic acid in the presence of carrier protein. Moreover, the percentage of acid-soluble radioactivity remained below 1.5% in those samples that were subsequently subjected to repeated freezing and thawing.

Human orosomucoid. Orosomucoid (7.7mg) was iodinated by the same procedure and volumes as described above for bovine serum albumin. The dialysed solution (1.0mg of glycoprotein/ml) contained only 0.7% of soluble radioactivity when the protein had been co-precipitated in the presence of carrier protein by a modified trichloroacetic acid method (see below). This value remained virtually constant during storage at -20°C. Treatment of the labelled orosomucoid solution with formaldehyde or urea was the same as for bovine serum albumin. Both solutions were stored at -20°C.

Desialylated human orosomucoid. Asialo-orosomucoid was iodinated by a similar procedure to that used for native human orosomucoid. The dialysed solution containing 0.64mg of ¹²⁵I-labelled asialo-orosomucoid/ml was stored at -20°C. In this solution only 0.9% of the total radioactivity remained soluble when the protein was precipitated in the presence of carrier albumin by the modified trichloroacetic acid method, and this value remained almost constant during storage.

Preparation and treatment of ¹²⁷I-labelled bovine serum albumin

A quantity of albumin was iodinated with (non-radioactive) [¹²⁷I]iodide, by using both the same number of g-atoms of iodide and the same procedure used in the preparation of the ¹²⁵I-labelled analogue. Portions of the preparation were exposed to the same chemical treatments as were described above for ¹²⁵I-labelled albumin.

Gel filtration

Each of the radioactively labelled protein samples prepared above was applied to a Sephadex G-200 column (1.8cm×56cm) and eluted with 0.05M-Tris/HCl buffer, pH 7.5, containing 0.1M-KCl, at a flow rate of 6ml/h (Andrews, 1964).

Uptake by rat visceral yolk sac cultured in vitro

Culture of yolk sac. Yolk sacs from 17.5-day-pregnant Wistar rats were incubated with labelled proteins at 37°C in medium 199 containing 10% (v/v) of calf serum by the method described in detail by Williams *et al.* (1975a,b). Approx. eight yolk sacs from the same rat were incubated, each in a separate flask, and removed sequentially from the incubating bath at approx. 1h intervals up to 7h. The associated culture flasks were all returned to the water bath and incubated until the last yolk sac was removed. This procedure was adopted to equalize any hydrolysis of labelled protein resulting from the presence of proteinase activity in the culture medium. Most experiments to determine the rate of uptake of radiolabelled proteins were performed in parallel with a second experiment with ¹²⁵I-labelled poly(vinylpyrrolidone) as the substrate. This substance has a well-defined rate of uptake (Williams *et al.*, 1975a), which acted as a measure of experimental reproducibility.

Assays of radioactivity. Both the yolk-sac tissue, dissolved in 5.0ml of aq. 1M-NaOH, and its associated culture medium were assayed by using duplicate 1.0ml portions of these solutions and a standard counting geometry (Roberts *et al.*, 1976). The amount of radiolabelled hydrolysis products in each sample of medium was assayed by precipitating the ¹²⁵I-labelled protein, centrifuging (30000g-min) and carefully decanting the clear supernatant for re-counting. Addition of trichloroacetic acid (0.5ml of 20%, w/v) gave quantitative precipitation of albumin, but, to achieve quantitative precipitation of ¹²⁵I-labelled orosomucoid and asialo-orosomucoid, it was necessary to add 0.5ml of phosphotungstate solution (Trinder, 1969) before addition of the trichloroacetic acid (0.5ml; 20%, w/v). The supernatant solutions (either 1.3 or 1.7ml in volume) were counted for radioactivity and the observed counts multiplied by the appropriate empirical factor required to normalize the count to that which would obtain if the same activity had been counted in a standard sample volume of 1.0ml.

Determination of protein. The protein content of each yolk sac was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as reference protein.

Expression of uptake rates. The rates of uptake of ¹²⁵I-labelled poly(vinylpyrrolidone) and ¹²⁵I-labelled proteins were expressed as Endocytic Indices (Williams *et al.*, 1975a,b). The associated calculations and the linear-regression analyses were performed with the aid of an ICL 4130 computer.

Uptake from the rat bloodstream in vivo

Male Wistar rats aged about 16 weeks and weighing 350–450g were anaesthetized with diethyl ether, and

a solution of ^{125}I -labelled protein (0.3 ml) was injected into the femoral vein at a dose of 0.3 mg of protein/kg body wt. Under continuous anaesthesia samples of blood (50 μl) were taken from the footpad of the hind leg on the opposite side to that injected. The first sample was taken approx. 60s after injection, and thereafter samples were taken at intervals throughout the 2h period of the experiment. Each was washed into a counting tube with a mixture of water (0.9 ml) and calf serum (0.1 ml), which provided carrier protein, and the contained radioactivity, assayed as described above, was designated the 'total radioactivity'. The appropriate precipitation procedure was then applied and the supernatant radioactivity ('acid-soluble radioactivity') measured and corrected for decreased counting efficiency resulting from increased sample volume (see above). The difference between these values gave the 'acid-insoluble radioactivity', a measure of the amount of undigested labelled protein in the bloodstream. The amounts of acid-soluble and acid-insoluble radioactivity in the blood were each expressed as a percentage of the amount of acid-insoluble radioactivity in the blood at zero time, as estimated by extrapolation.

After 2h the rat was killed and the liver freed from blood by flushing it *in situ* via the hepatic vein with ice-cold 1% NaCl (20ml). The whole liver was then removed and weighed. A weighed portion (approx. 1g) was assayed for radioactivity, enabling the total radioactivity in the whole liver to be expressed as a percentage of the amount of acid-insoluble radioactivity that had been injected.

Results

Gel filtration

Each of the ^{125}I -labelled albumin preparations was resolved by Sephadex G-200 into a major component, with an elution position indicative of monomeric material (mol.wt. approx. 66000), and a minor (approx. 5%) component corresponding to the dimer. Another small band appeared at the elution position of [^{125}I]iodide. The ^{125}I -labelled samples of both orosomucoid and asialo-orosomucoid were eluted as single bands at an elution volume consistent with the presence of monomeric material (mol.wt. 44000); a small amount of [^{125}I]iodide was present, but there was no evidence of oligomer formation.

Uptake by rat visceral yolk sac cultured *in vitro*

Control experiments (17), with ^{125}I -labelled poly-(vinylpyrrolidone) as substrate at a concentration of 1 $\mu\text{g}/\text{ml}$ of medium, were performed at regular intervals throughout the whole period of study. The absence of any systematic drift in the individual values of the Endocytic Index [mean value

Fig. 1. Accumulation of radioactivity by yolk-sac tissue and appearance of trichloroacetic acid-soluble radioactivity in the culture medium, on incubation of yolk sacs in the presence of formaldehyde-treated ^{125}I -labelled bovine serum albumin

Points at each time-interval correspond to data derived from a single yolk sac incubated separately for up to 6.0h. The ordinate shows the volume of culture medium whose content of trichloroacetic acid-insoluble radioactivity is found associated with the tissue in either trichloroacetic acid-insoluble or -soluble form (●), or is found in the culture medium as trichloroacetic acid-soluble radioactivity (○).

1.5 ± 0.2 (s.d.) $\mu\text{l}/\text{h}$ per mg of yolk-sac protein] indicated the high degree of reproducibility of pinocytic activity in the tissue-culture system.

Fig. 1 shows a typical experiment in which yolk sacs were incubated in the presence of ^{125}I -labelled bovine serum albumin. The amount of radioactivity found in the tissue and the amount of acid-soluble radioactivity released back into the culture medium are both shown. Acid-soluble radioactivity appeared in the medium at a constant rate, and the specific radioactivity associated with the yolk sac was effectively constant. This pattern was seen with all preparations of albumin and is in agreement with previous observations (Williams *et al.*, 1975b). The overall rate of uptake can be calculated by summing, at each time-interval, the acid-soluble radioactivity present in the medium and the total radioactivity associated with the tissue. Fig. 2 shows data relating to two different albumin preparations, calculated in this way. Examination of the results of each individual experiment with the various ^{125}I -labelled albumin preparations revealed that in all cases uptake was linear with time. In all experiments the

correlation coefficient associated with the straight line fitted by linear-regression analysis was in the range 0.90–0.99. The results are summarized in Table 1, which also includes the Endocytic Index of

Fig. 2. Uptake of ^{125}I -labelled albumin preparations by yolk sacs incubated *in vitro*

The ordinate axis shows the total volume of culture medium whose contained ^{125}I -labelled albumin has been ingested by the tissue (calculated by summing the total radioactivity in the tissue and the acid-soluble radioactivity released back into the medium). ●, Formaldehyde-treated ^{125}I -labelled albumin (same data as shown in Fig. 1); ○, non-frozen ^{125}I -labelled albumin. The gradient of each plot gives a single value of the Endocytic Index.

^{125}I -labelled poly(vinylpyrrolidone). Each preparation of albumin showed a highly reproducible and characteristic Endocytic Index. Moreover, in cases where more than one batch of a particular preparation had been produced, there was no significant interbatch variation in Endocytic Index; results have therefore been pooled.

All the ^{125}I -labelled albumin preparations showed Endocytic Indices significantly greater than that of ^{125}I -labelled poly(vinylpyrrolidone). Freezing the ^{125}I -labelled albumin preparation, or exposing it to NaHCO_3 buffer, pH 10, increased the rate of uptake to approximately twice that of the non-frozen preparation. The various acid treatments gave a further increase, but the most marked increases were produced by exposure to either formaldehyde

Table 2. Effect of the presence of variously treated (non-radioactive) ^{127}I -iodinated bovine serum albumin preparations on the rate of uptake of ^{125}I -labelled poly(vinylpyrrolidone) by rat yolk sac cultured *in vitro*

Rates of uptake are expressed as Endocytic Indices (see the text). Each value of the Endocytic Index was derived from the plot of uptake against time by using data from a single experiment, as in Table 1. In each experiment both ^{127}I -iodinated bovine serum albumin (1 μg/ml) and ^{125}I -labelled poly(vinylpyrrolidone) were added to the culture medium.

Preparation of ^{127}I -iodinated bovine serum albumin	Endocytic Index of ^{125}I -labelled poly(vinylpyrrolidone) (μl/h per mg of yolk-sac protein)
Untreated, stored at 4°C	2.0
Acetic acid, pH 3.5	1.3
Acetic acid, pH 3.0	1.3
Acetic acid, pH 2.5	1.6
Formaldehyde, pH 10	1.5

Table 1. Rates of uptake of various preparations of ^{125}I -labelled bovine serum albumin and ^{125}I -labelled poly(vinylpyrrolidone) by rat yolk sacs cultured *in vitro*

Rates of uptake are expressed as Endocytic Indices (see the text). Each value of the Endocytic Index was derived from the plot of uptake against time by using data from an experiment in which six to ten 17.5-day yolk sacs from a single animal were incubated for intervals up to a maximum of 7 h. The amount of radioactivity in the tissue, expressed in the same units as uptake, is also shown. Results are expressed as means $\pm$ S.D. for the numbers of experiments stated.

Substrate preparation	No. of batches used	No. of experiments	Endocytic Index (μl/h per mg of yolk-sac protein)	Tissue radioactivity (μl/mg of yolk-sac protein)
^{125}I -labelled poly(vinylpyrrolidone)	5	17	1.5 $\pm$ 0.2	—
^{125}I -labelled bovine serum albumin:				
Untreated, stored at 4°C	3	6	4.8 $\pm$ 1.5	6.5 $\pm$ 2.5
Freezing, -20°C	1	5	8.9 $\pm$ 2.0	7.0 $\pm$ 1.0
Acetic acid, pH 3.5	2	10	16.8 $\pm$ 2.6	17.0 $\pm$ 4.5
Acetic acid, pH 3.0	1	4	23.4 $\pm$ 4.0	16.5 $\pm$ 3.0
Acetic acid, pH 2.5	1	4	14.5 $\pm$ 3.1	12.5 $\pm$ 4.0
Formaldehyde, pH 10	1	4	65 $\pm$ 11	100 $\pm$ 33
Buffer, pH 10	1	1	8.1	7.0
Urea, pH 5.5	1	3	73.3 $\pm$ 6.7	65 $\pm$ 7

Table 3. *Rate of uptake of various preparations of ^{125}I -labelled human orosomucoid by 17.5-day rat yolk sacs cultured in vitro*
Rates of uptake are expressed as Endocytic Indices (see the text) derived as explained in Table 1. The amount of radioactivity in the tissue is also shown. Where appropriate, results are given as means $\pm$ s.d. for the numbers of experiments stated.

Substrate preparation	No. of experiments	Endocytic Index ($\mu\text{l/h}$ per mg of yolk-sac protein)	Tissue radioactivity ($\mu\text{l/mg}$ of yolk-sac protein)
^{125}I -labelled orosomucoid:			
Untreated, stored at 4°C	5	6.1 ± 1.7	9.0 ± 3.5
Urea, pH 5.5	1	5.5	8.0
Buffer, pH 10	1	4.8	8.0
Formaldehyde, pH 10	1	2.2	8.0
^{125}I -labelled asialo-orosomucoid:			
Untreated, stored at 4°C	3	12.5 ± 1.1	18.0 ± 7.0

or urea, both of which increased the rate of uptake to more than 10 times that of the unfrozen preparation. The rate of accumulation of ^{125}I -labelled poly(vinylpyrrolidone) was unaltered by the presence of variously treated (non-radioactive) [^{127}I]iodinated albumins ($1 \mu\text{g/ml}$) in the culture medium (Table 2).

Table 3 shows the results of experiments with ^{125}I -labelled orosomucoid and asialo-orosomucoid. In each case uptake was linear with time and the rate of uptake was reproducible between experiments. A non-frozen ^{125}I -labelled preparation of orosomucoid showed a low Endocytic Index similar to that of untreated ^{125}I -labelled albumin; exposure to urea or buffer, pH 10, had little effect, and exposure to formaldehyde caused a decrease in Endocytic Index. Insufficient ^{125}I -labelled asialo-orosomucoid was available to permit study of its chemically modified forms, but the non-frozen preparation showed a rate of uptake only twice that of non-frozen ^{125}I -labelled orosomucoid.

Uptake from the rat bloodstream in vivo

Typical clearance patterns of the different ^{125}I -labelled albumin preparations from the bloodstream over a 2 h period are shown in Fig. 3. The clearance pattern for each preparation was reasonably reproducible from one animal to another. The clearance of 90% of the formaldehyde-treated bovine serum albumin within 1 h is in agreement with the observations of Buys *et al.* (1973). The corresponding quantities of acid-soluble radioactivity appearing in the bloodstream over the same period, in the same experiments, are shown in Fig. 4; the concentration of acid-soluble radioactivity never much exceeded 7% of the initial concentration of injected protein.

Similar data are shown in Fig. 5 for the clearance of ^{125}I -labelled orosomucoid and asialo-orosomucoid. ^{125}I -labelled orosomucoid was cleared at approximately twice the rate of untreated ^{125}I -labelled albumin. With ^{125}I -labelled asialo-orosomucoid the amount of acid-insoluble radioactivity in the

Fig. 3. *Clearance of ^{125}I -labelled albumin preparations from the bloodstream*

The amount of acid-insoluble radioactivity in the bloodstream at a given time is expressed as a percentage of that present in the bloodstream immediately after intravenous injection of a particular preparation of ^{125}I -labelled albumin; $\blacktriangle$, non-frozen; $\circ$, acetic acid-treated (pH 2.5); $\blacksquare$, urea-treated; $\bullet$, formaldehyde-treated. Each plot shows the data from a representative experiment with a single animal.

bloodstream fell rapidly to reach a constant value of approx. 3% of the initial value after 30 min and is consistent with the clearance pattern reported by Ashwell & Morell (1974). The associated amounts of trichloroacetic acid-soluble radioactivity in the blood are shown in Fig. 6. With asialo-orosomucoid the amount of radioactivity rose progressively over the first hour in a similar manner to that observed with formaldehyde-treated albumin, but reached a slightly higher value (approx. 10%).

Fig. 4. Appearance of acid-soluble ^{125}I -labelled hydrolysis products in the bloodstream after injection of different ^{125}I -labelled albumin preparations

The amount of acid-soluble radioactivity in the bloodstream at a given time is expressed as a percentage of the acid-insoluble radioactivity present in the bloodstream immediately after injection of a particular preparation of ^{125}I -labelled albumin. The experiments are the same as shown in Fig. 3: ▲, non-frozen; ○, acetic acid-treated (pH2.5); ■, urea-treated; ●, formaldehyde-treated.

Fig. 5. Clearance from the bloodstream of orosomucoid and asialo-orosomucoid, each labelled with ^{125}I iodide. Data are expressed as indicated in the legend to Fig. 3: ○, orosomucoid; ●, asialo-orosomucoid. Each plot shows the data from a representative experiment in which blood samples ($50\mu\text{l}$) were taken from a single animal.

Table 4 shows the amounts of radioactivity present in the liver 2h after injection of labelled protein; the concentration of acid-soluble and acid-insoluble radioactivity in the blood at this time are also shown.

Fig. 6. Appearance of acid-soluble ^{125}I -labelled hydrolysis products in the bloodstream after injection of either ^{125}I -labelled orosomucoid or asialo-orosomucoid

Data are expressed as indicated in the legend to Fig. 4: ○, orosomucoid; ●, asialo-orosomucoid.

Discussion

Much of the following discussion, particularly that relating to experiments with rat yolk sac incubated *in vitro*, rests on the assumption that the protein hydrolysis observed occurs exclusively intracellularly and within the lysosomal system. In the culture system the amount of protein hydrolysed by free proteinase activity present in the culture medium was shown to be negligible, but nevertheless the method used would correct for any hydrolysis from this origin. But protein hydrolysis could also occur by neutral proteinases associated with the microvillus membrane of the yolk sac. A decisive experiment to differentiate extracellular from intracellular proteolysis has so far proved impossible to devise, but several pieces of circumstantial evidence indicate that the observed hydrolysis takes place after pinocytic uptake of protein, i.e. is intracellular. Firstly, a preliminary investigation of peptidase activity in plasma membrane-rich fractions of rat yolk sac showed very low specific activities (A. J. Kenny, unpublished work) in comparison with similar fractions from rabbit kidney proximal tubules (Kerr & Kenny, 1974). Secondly, subcellular fractionation of rat yolk sac that had taken up ^{125}I -labelled serum albumin *in vivo* (Williams *et al.*, 1971) or *in vitro* (Goetze *et al.*, 1976) indicated concentration within the lysosomes. Thirdly, the rate of accumulation of the non-hydrolysed macromolecule ^{125}I -labelled poly(vinylpyrrolidone) and the rate of hydrolysis of several ^{125}I -labelled proteins, in the yolk-sac system, are affected to a comparable extent both by an inhibitor of pinocytosis, Trypan Blue (Williams *et al.*, 1973), and by a decrease in the temperature at which the culture is performed

Table 4. Distribution of ^{125}I radioactivity 2.0h after injection of tracer quantities of ^{125}I -labelled preparations of albumin, orosomucoid or asialo-orosomucoid into male rats

The ^{125}I -labelled proteins were each injected into the femoral vein of a male Wistar rat (350–450g) at a dose of 0.3–0.9mg/kg body wt. After removal of blood samples (50 μl) at regular intervals, the animal was killed at 2.0h and the residual radioactivity in the blood and liver was compared with the amount of trichloroacetic acid-insoluble radioactivity injected. Each line of the Table shows the results of a single representative experiment.

Substrate preparation	Radioactivity found in different locations at 2.0h (% of acid-insoluble radioactivity injected)			
	Acid-insoluble radioactivity in blood	Acid-soluble radioactivity in blood	Total radio- activity in liver	Radioactivity not accounted for
^{125}I -labelled bovine serum albumin:				
Untreated, stored at 4°C	82	1	2	15
Acetic acid, pH 2.5	75	2	5	18
Formaldehyde, pH 10	8	7	12	73
Urea, pH 5.5	52	3	3	42
^{125}I -labelled orosomucoid:				
Untreated, stored at 4°C	55	5	8	32
^{125}I -labelled asialo-orosomucoid:				
Untreated, stored at 4°C	3	11	13	73

(G. Livesey & K. E. Williams, unpublished work), indicating that the same process (pinocytosis) is affected in both cases. Fourthly, the quantitative similarity of the Endocytic Indices of ^{125}I -labelled poly(vinylpyrrolidone) and albumins (particularly in undenatured form) would be totally unexpected if the former represented a rate of pinocytosis and the latter a kinetic characteristic of some extra-cellular proteinase.

The results in Table 1 indicate that the various treatments of ^{125}I -labelled albumin increased its rate of uptake by the rat yolk sac *in vitro* relative to the rate of uptake of unfrozen ^{125}I -labelled albumin. For all these albumin preparations (and for the glycoproteins used), the amount of radioactivity associated with the yolk-sac tissue rose initially, but after 1.5–2h reached a constant value (see Fig. 1). A steady-state concentration within the tissue indicates that the tissue degrades exogenous protein at a rate equal to that of its capture; thus pinocytic ingestion is the rate-limiting step, and therefore a potential control point in the overall process of ingestion and catabolism.

Several explanations are possible for the higher rates of pinocytic uptake of modified albumins. The substrates themselves might stimulate the rate of pinosome formation, but this explanation is excluded by the observation (Table 2) that no increase in the rate of uptake of ^{125}I -labelled poly(vinylpyrrolidone) was caused on adding to the culture medium tracer amounts of non-radioactive ^{127}I -iodinated analogues of the treated albumins. Alternatively several distinct modes of endocytosis might be operative in the yolk sac, each available only to certain substrates. Indeed ultrastructural studies have indicated the existence in rabbit yolk-sac epithelial cells of two classes of

vesicle, one that fuses and one that fails to fuse with lysosomes. The second type, the coated micropinocytic vesicles, appears to be specific for uptake of homologous γ -globulin and to transport it intact across the cell (Moxon & Wild, 1976). One could postulate the existence of morphologically indistinguishable subclasses of non-coated pinocytic vesicles, but there is no supporting evidence and it would be absurd to propose a distinct type of vesicle for each preparation of albumin investigated. The third possibility is that increased adsorption to the plasma membrane, from which pinocytic vesicles form, results from aggregation or modified conformation of the protein.

Sherman *et al.* (1974) reported that human serum albumin can aggregate on iodination if the chloramine-T:albumin ratio is in excess of 2:1 (w/w). In the present study iodination was with a low chloramine-T:protein ratio (1:5, w/w) and chromatography on Sephadex G-200 indicated that the extent of aggregation, both in the stock ^{125}I -labelled albumin solution and in portions of it that were further treated with chemical reagents, was no more than 5%. However, even this degree of heterogeneity could invalidate deductions from the rate of protein uptake if a small fraction only of the labelled protein present in the culture medium was captured during the incubation period. In our experiments an Endocytic Index of 50 $\mu\text{l}/\text{h}$ per mg of yolk-sac protein corresponds to ingestion of the available tracer protein at a rate of approx. 3%/h, so that over the 6–7h duration of an experiment 20% of the protein is internalized. With all the iodinated albumin preparations studied, the rate of uptake was constant throughout the incubation period, with no initial phase of rapid uptake indicative of the presence of a small percentage of

some species that is ingested preferentially. Since the protein preparations had no more than 5% of non-monomeric forms aggregation cannot be the cause of the increased clearance rates *in vitro*. It is concluded that more subtle changes in the conformation of albumin molecules are responsible, by increasing the extent of binding to the plasma membrane.

In the present investigation preliminary studies of the optical-rotatory-dispersion and circular-dichroism spectra indicated that ^{125}I -iodinated albumin that had received no additional chemical treatment has the same degree of helical character (40–50%) as native albumin; hence iodination by the chloramine-T method is unlikely to induce a major conformational change. The same conclusion was reached by Buys *et al.* (1975) who determined the content of reducible disulphide groups in both ^{125}I -iodinated bovine serum albumin (labelled by a chloramine-T method similar to that used in the present work) and native bovine serum albumin and found similar low values.

Formaldehyde-treated bovine serum albumin, prepared virtually as in the work reported here, contained some dimer, whereas the monomer had an increased Stokes radius attributable to an unfolding of the tertiary structure (Buys *et al.*, 1973). This explanation is concordant with the later observation of a marked increase in the number of reducible disulphide groups in the protein on exposure to formaldehyde (Buys *et al.*, 1975). A preliminary investigation of the conformational states of the various preparations of bovine serum albumin by optical rotatory dispersion and circular dichroism (see Lloyd *et al.*, 1975) suggested, qualitatively, that treatment with urea or formaldehyde caused extensive loss of the α -helical content of the molecule. Presumably modification of albumin with either of these reagents greatly increases its affinity for plasma-membrane binding sites.

Several authors (see Franglen, 1974) report that bovine serum albumin undergoes a conformational change in the region of pH 4 with formation of an expanded structure of greater electrophoretic mobility. This conformational change, unlike that induced by formaldehyde, is reported to be freely and rapidly reversible, so that the raised Endocytic Index of the dialysed acetic acid-treated albumin (see Table 1) was unexpected. Acid treatment may cause small, but irreversible, conformational changes not readily detected by physical methods, but important in determining the affinity of a protein for the plasma membrane.

The more limited series of experiments with ^{125}I -labelled orosomucoid (see Table 3) showed that treatment with urea caused no change in Endocytic Index. This finding may well reflect the known ability of this glycoprotein to resist denaturation, even by boiling (Poortmans, 1962). Exposure to formaldehyde decreased the Endocytic Index to a

value similar to that of ^{125}I -labelled poly(vinylpyrrolidone). This result contrasts sharply with that seen with albumin.

From the above studies it is apparent that small changes in protein conformation or charge may be sufficient to modify the extent of interaction between a protein and binding sites on the plasma membrane. Currently nothing is known at the molecular level of the nature of these interactions or even which components of the plasma membrane are involved. Specific charged or polar amino acid residues at the surface of the protein may be involved in the interaction with protein or glycoprotein components of the plasma membrane, a possibility suggested by the observation (Buys *et al.*, 1975) that treatment of albumin with formaldehyde blocks approx. 50% of the lysine side chains. The isolation, by Hudgin *et al.* (1974) of a glycoprotein from rabbit liver that selectively binds asialo-glycoprotein *in vitro* supports the potential involvement of glycoproteins of the plasma membrane in the binding process. Alternatively, hydrophobic regions of the surface of the protein may interact with lipid elements of the plasma membrane. Clearly, further work is necessary to examine these possibilities.

The Endocytic Indices of the different protein preparations give some indication of the extent to which their ingestion by pinocytosis is dependent on binding to plasma membrane, since the Endocytic Index of ^{125}I -labelled poly(vinylpyrrolidone) gives an upper limit to the amount of substrate that enters in the liquid phase. If the Endocytic Index of ^{125}I -labelled poly(vinylpyrrolidone) is taken as $2\mu\text{l/h}$ per mg of protein, a preparation of protein with an Endocytic Index of $50\mu\text{l/h}$ per mg of protein must be internalized at least 96% by adsorption and only 4% in the bulk liquid phase. Of course the ingestion of ^{125}I -labelled poly(vinylpyrrolidone) itself may be to some extent by adsorption and, if this is the case, the percentage of the protein taken up by adsorption will be even higher.

The results presented in Fig. 3 show that after intravenous injection, the formaldehyde- and urea-treated preparations of ^{125}I -labelled albumin are each cleared from the bloodstream at much higher rates than untreated ^{125}I -labelled albumin. Similarly, the results of Fig. 5 confirm the work of Ashwell & Morell (1974) in showing a dramatic increase in the rate of clearance of ^{125}I -labelled orosomucoid on removal of terminal sialic acid residues. The observed reappearance of radioactivity in the bloodstream in acid-soluble form (see Figs. 4 and 6) is compatible with the findings, by subcellular fractionation techniques, that formaldehyde-treated ^{131}I -labelled albumin (Bertini *et al.*, 1967), ^3H -labelled asialoceruloplasmin (Gregoriadis *et al.*, 1970) and ^{125}I -labelled asialofetuin (LaBadie *et al.*, 1975) all entered the lysosomes of liver cells after clearance from the

bloodstream. Within 1.0h of injection of ^{125}I -labelled asialo-orosomucoid the concentration of acid-soluble radioactivity in the blood exceeds that of acid-insoluble radioactivity (Figs. 5 and 6); in consequence, studies of rapidly cleared proteins in which acid-soluble and acid-insoluble radioactivities are not differentiated are likely to be in serious error.

Fig. 5 shows that ^{125}I -labelled asialo-orosomucoid is completely cleared from the bloodstream within 15min, and Morell *et al.* (1971) have reported that 98% of their injected ^3H -labelled asialo-orosomucoid was recovered from the liver at 20min. The finding (Table 4), with ^{125}I -labelled asialo-orosomucoid, that approx. 13% of the ^{125}I injected was still associated with the liver 2.0h after injection, suggests that intracellular catabolism, rather than uptake, by the hepatocyte is the rate-limiting step in the overall process of uptake and digestion. Essentially the same pattern was also observed with formaldehyde-treated albumin (Table 4), suggesting that in the Kupffer-cell protein hydrolysis is again the rate-limiting step. It is noteworthy that for these two rapidly cleared proteins, over 70% of the injected radioactivity cannot be accounted for at 2h in either the liver or the blood. Presumably, most of the acid-soluble radioactivity released from the liver has by 2h been removed from the bloodstream by the kidneys.

When the individual preparations of ^{125}I -labelled albumin are ranked according to their clearance rates *in vivo*, the resulting ranking closely parallels that based on the Endocytic Indices of these same preparations when ingested by rat yolk sac *in vitro*. This suggests that for some protein preparations the conformational change generates a relatively non-specific determinant that is similarly recognized by more than one cell type. Comparison of the rates of clearance of ^{125}I -labelled orosomucoid and asialo-orosomucoid *in vivo* and *in vitro* indicates that the opposite can also be true. Removal of terminal sialic acid from orosomucoid generates a determinant that is recognized clearly by the hepatic parenchymal cell, but by neither the Kupffer cell nor the yolk-sac epithelial cell (see Table 3), indicating that structural differences in the plasma membrane are also important in the overall recognition process.

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