THE FATE OF INTRAVENOUS DOSES OF FREE AND PLASMA PROTEIN-BOUND [^{35}S]CYSTINE IN THE SHEEP

By A. M. Downes*

[*Manuscript received January* 23, 1961]

Summary

When a tracer quantity of L- or DL-[^{35}S]cystine was mixed with sheep blood plasma about two-thirds of the radioactivity was bound to the proteins after 4 hr at 39°C, probably as half-cystine residues in disulphide linkage. The reversibility of the binding was demonstrated.

Intravenous doses of L-[^{35}S]cystine, both as the free amino acid and as half-cystine residues bound by disulphide bonds to plasma proteins *in vitro*, were given to sheep, and the fate of the ^{35}S studied. The results suggested that at least some of the cystine not in peptide linkage is bound to the plasma proteins *in vivo* and that there is a continuous exchange between the free and bound forms with a half-time of about 1 hr. It was not possible to decide from these experiments whether the free or the disulphide-bound cystine play any special role in keratinization.

On the average, about 30% of an intravenous dose of L-[^{35}S]cystine appeared in the wool grown during the 28 days after the dose, whether the cystine was given in the free or the disulphide-bound form or whether the specific activity was lowered by the dilution of the dose with as much as 2 g of "carrier" L-cystine. The significance of these results is discussed.

I. Introduction

During a previous study of the mechanism of incorporation of [^{35}S]cystine into wool (Downes 1961) it was found that a large proportion of the radioactivity became bound to the plasma proteins *in vitro*. Cystine added to plasma evidently equilibrates rapidly with free cysteine and cystine (cyst(e)ine) and with half-cystine residues held by disulphide bonds by reaction with the –SH groups of the protein molecules. The bound cystine is not removed by precipitation of the proteins with trichloroacetic acid but is removed by treatment with reducing agents such as mercaptoethanol (Lee *et al.* 1951) or $NaHSO_3$ (Downes 1961). If there is significant binding of cyst(e)ine to plasma proteins *in vivo* it would be important to know if the bound form plays any special role in the synthesis of wool keratin. For example it is possible that cyst(e)ine has to be carried in the bound form to the skin or the wool follicles to be extracted by disulphide interchange.

Further studies on the binding of cystine by plasma proteins *in vitro* have now been made. Other experiments described here were carried out to see if the binding of cystine by the plasma proteins occurs *in vivo* and to study the fate of the bound cystine in the sheep. The efficiency of incorporation into wool of intravenous doses of L-[^{35}S]cystine diluted with various amounts of non-radioactive L-cystine was also measured.

* Division of Animal Physiology, C.S.I.R.O., Ian Clunies Ross Animal Research Laboratory, Prospect, N.S.W.

II. Materials and Methods

The doses of L-[^{35}S]cystine (from the Abbott Laboratories, Chicago, U.S.A.) and of DL-[^{35}S]cystine (from the Radiochemical Centre, Amersham, England) were prepared and the radiochemical purity checked as described previously (Downes and Lyne 1961).

The experimental animals (three Corriedale wethers and one ewe) were kept in metabolism cages in an animal house and were fed a constant daily ration of 800–1000 g of equal parts of lucerne and wheaten chaff.

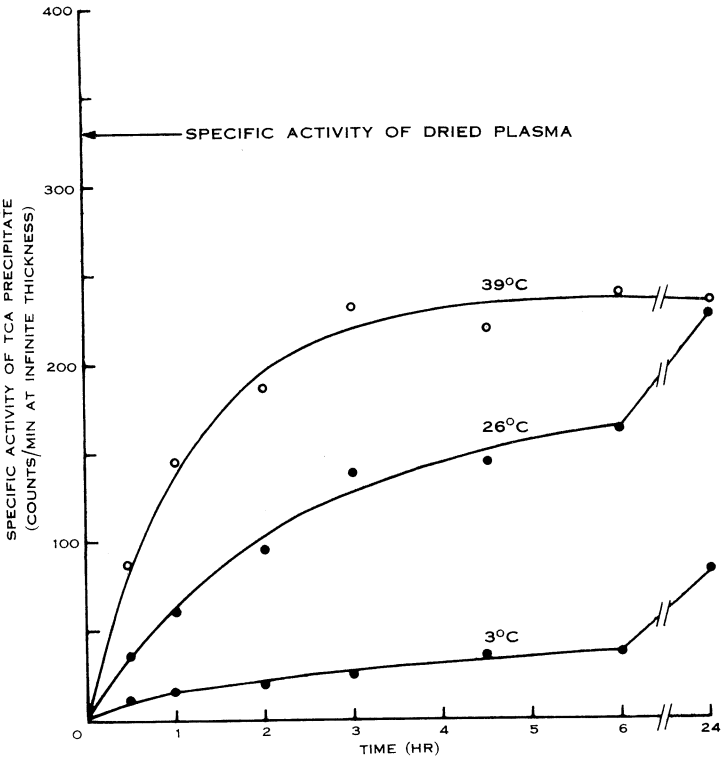

Fig. 1.—Rate of binding of [^{35}S]cystine by plasma proteins at different temperatures. To each of six 10-ml samples of plasma freshly obtained from a sheep was added 0·1 ml of aqueous L-[^{35}S]cystine (16 μg; 0·31 μc) as the hydrochloride. Two of the mixtures were kept at 39°C, two at 26°C, and two at 3°C. Samples were removed at intervals and the proteins precipitated with TCA. The results are the means of each pair of duplicates. The specific activity of the dried plasma is also shown to indicate the degree of binding.

The methods of radioassay and of blood analysis were those described by Downes (1961) except that the plasma proteins were washed with acetone, instead of with ethanol and ether, after precipitation with trichloroacetic acid (TCA). The ^{35}S present in TCA precipitates of plasma which had been pretreated with $NaHSO_3$ was assumed to be "peptide-bound ^{35}S", that is with the cystine present in peptide linkage. The extra ^{35}S in TCA precipitates of untreated plasma is

referred to as "disulphide-bound ^{35}S", presumably present as half-cystine residues bound by disulphide bonds.

The wool samples were cleaned by washing with ether, ethanol, and water, and were heated at 110°C for 18 hr. Samples were analysed for sulphur by the method of Myers (1959). Other samples were oxidized by the method of Myers (1959) and counted as benzidine sulphate at "infinite" thickness. All measurements of ^{35}S were referred to the counting rates of standards prepared from the labelled cystine.

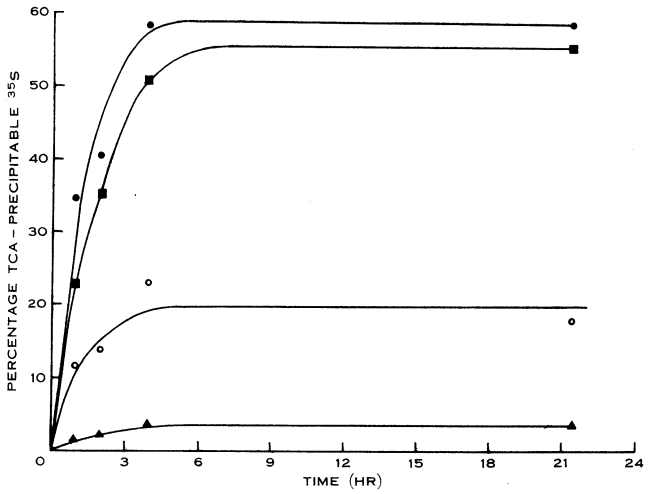

Fig. 2.—Effect of carrier cystine on the binding of [^{35}S]cystine by plasma proteins. To each of four 8-ml portions of sheep plasma at 39°C was added 0·2 ml of a solution of DL-[^{35}S]cystine hydrochloride. Samples were removed at 1, 2, 4, and 21·5 hr and the proteins precipitated with TCA. The amounts of cystine used and their specific activities were:

● 0·040 mg, 0·68 μc ○ 1·51 mg, 0·68 μc
■ 0·160 mg, 2·70 μc ▲ 11·1 mg, 0·68 μc

III. Experimental Results

(*a*) *Binding of Cystine by Plasma Proteins* in vitro

Solutions of [^{35}S]cystine hydrochloride were incubated at various temperatures with blood plasma freshly obtained from sheep. This treatment altered the pH of the plasma by no more than 0·05 units. The binding was followed by removing samples (1 ml) at intervals and precipitating the proteins with TCA (4 ml, 10% w/v). The precipitates were washed three times with TCA (5% w/v) and several times with acetone, dried, and their specific activities measured.

The results in Figures 1 and 2 show that ^{35}S rapidly became bound to the plasma proteins *in vitro*. At 39°C an equilibrium appeared to be established in about 4 hr but at lower temperatures the rate of binding was slower. From Figure 2 it may be seen that the larger the amount of cystine added the smaller was the percentage

of bound ^{35}S. With very small amounts of added cystine (20 and 5 μg per ml plasma) practically the same percentage was obtained, showing that the amount of free plus bound cystine in the plasma must be much larger than 20 μg/ml. As shown in Figure 3 the further addition of a relatively large mass of non-radioactive cystine removed a large proportion of the bound ^{35}S.

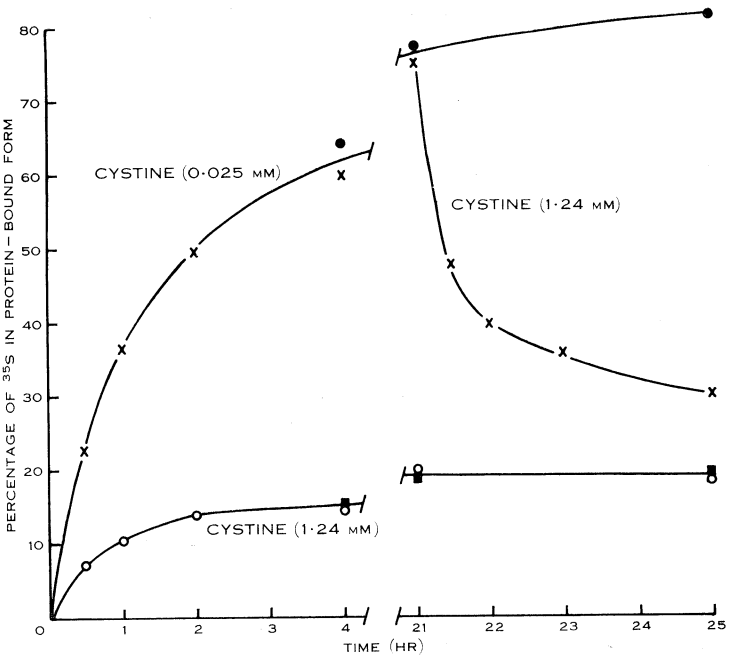

Fig. 3.—Demonstration of the reversibility of binding of L-[^{35}S]cystine by sheep plasma proteins Four samples of plasma were heated at 39°C with L-[^{35}S]cystine and 1-ml portions removed at intervals for the measurement of bound ^{35}S. Two of the solutions contained added cystine at a concentration of 1·24 mM; in the other two the concentration was 0·025 mM. After 21 hr one of the latter solutions was also made 1·24 mM with respect to added cystine and the sampling continued for a further 4 hr.

From Table 1 it may be seen that the bound ^{35}S was not removed by dialysis overnight against water and that the same results were obtained when picric acid was used as the precipitant instead of TCA. Other experiments, in which the proteins were similarly labelled, showed that 30–70% of the bound radioactivity was lost when the proteins were coagulated by boiling the solutions for a few minutes.

At equilibrium about 60–80% of the radioactivity was precipitable with TCA when tracer amounts of cystine were added. DL-[^{35}S]cystine was similarly mixed with fresh plasma (about 1 μg cystine per ml plasma) from several other species. In each case curves similar to those in Figures 1 and 2 were obtained. The species used and the percentage of ^{35}S precipitable with TCA after 24 hr at 20°C were: man 11, cow 51, domestic fowl 6, rat 78, rabbit 71, and brush-tailed possum 43.

(*b*) *Binding of Cystine by Plasma Proteins* in vivo

L-[^{35}S]cystine (356 μc; 6·7 mg) was injected intravenously into a Corriedale ewe. Blood samples were taken at intervals, cooled immediately to below 5°C, and centrifuged at 2700 *g* for 30 min at 5°C. The plasma was then analysed as soon as possible for total ^{35}S, peptide-bound ^{35}S, disulphide-bound ^{35}S, free cystine ^{35}S, and sulphate ^{35}S. The proteins were precipitated no later than 1 hr after the blood samples were taken.

The changes which occurred in the distribution of ^{35}S in the blood plasma during the first 7 hr are shown in Figure 4. Since the plasma samples were processed

TABLE 1

SPECIFIC ACTIVITIES OF PROTEIN SAMPLES OBTAINED IN THREE WAYS FROM PLASMA–[^{35}S]CYSTINE MIXTURES

Sheep plasma (5 ml) and L-[^{35}S]cystine (0·19 μc; 16 μg in 0·5 ml 0·9% NaCl) were mixed and heated at 39°C for 6 hr. Duplicate samples of the proteins were then obtained by the methods listed and their specific activities measured. The counting rate of the dried plasma is included to indicate approximately the maximum specific activity possible

Sample	Specific Activity (counts/min at infinite thickness)	Sample	Specific Activity (counts/min at infinite thickness)
Dried plasma	333 321	TCA precipitate of sample dialysed overnight against running tap water	240 223
TCA precipitate	222 242	Picric acid precipitate*	252 228

* Obtained by method of Stein and Moore (1954).

immediately and kept cold (5°C) throughout, it was estimated from Figure 1 that no more than 10% of the free cystine-^{35}S in each sample could have become bound to the proteins during the processing. Nevertheless the amount of non-peptide radioactivity in the bound form increased to about 70% during the first hours after the injection. The absolute amount of bound ^{35}S was maximal about 15 min after the injection and then declined approximately exponentially with a half-time of about 2 hr.

Similar results were obtained when this experiment was repeated with another sheep. On this occasion some of the TCA precipitate (281 mg) of plasma from a blood sample taken 1 hr after the injection was oxidized with performic acid, hydrolysed, and the cysteic acid isolated from the hydrolysate as described previously (Downes 1961). The TCA precipitate contained 55% of its radioactivity as peptide-bound ^{35}S and the cysteic acid recovered accounted for at least 80% of the initial ^{35}S. Since some losses undoubtedly occurred during the above procedure it is evident that the bulk of the initial ^{35}S was present as half-cystine residues.

(c) *Efficiency of Incorporation of* L-[^{35}S]*Cystine into Wool*

Three Corriedale wethers were given a series of intravenous injections of L-[^{35}S]cystine, as the free amino acid or in the partly disulphide-bound form.

A typical dose of plasma-containing disulphide-bound L-[^{35}S]cystine was prepared as follows. About 100 ml blood was collected using heparin (1 ml, 1% w/v) as the anticoagulant, and immediately centrifuged for 40 min at 600 *g* and 5°C. The bulk of the plasma was transferred to a flask containing L-[^{35}S]cystine (28·2 μc; 540 μg cystine as the hydrochloride in 0·126 g aqueous solution). The mixture was kept for 8 days at 5°C (to avoid growth of bacteria in case of accidental contamination) and then injected intravenously into the same sheep. In the experiments with sheep MA51 and MA52 appropriate precautions were taken to keep the plasma and the L-[^{35}S]cystine sterile throughout. Just before each injection a sample of the labelled plasma was taken for the estimation of the percentage of disulphide-bound ^{35}S. In each case this was about 60% of the total ^{35}S. Samples of the mixture injected into MA51 and MA52 were also taken for bacteriological examination and found to be sterile. Two of the sheep (MA50, MA52) received the labelled plasma first and a dose of free L-[^{35}S]cystine about 4 weeks later. This order was reversed in sheep MA51.

The importance of the specific activity of the doses of free cystine was determined by giving each of the three sheep another dose of free L-[^{35}S]cystine diluted with 0·10, 0·50, and 2·0 g respectively of carrier.

The fleece was clipped as completely as possible just before each injection. Two tattooed areas, one on each side of the sheep, were clipped weekly. The mass of clean dry wool and the specific activity of the benzidine sulphate derived from each sample were measured. Blood samples were taken during the 6 hr after each injection and the specific activity of samples of the dried plasma was measured.

The doses and amounts of ^{35}S incorporated into the wool are summarized in Table 2. To illustrate the reproducibility of the results and the method of calculation, the details of two of the experiments with sheep MA51 are given in Table 3. Corrections were made for the contribution to the total radioactivity from the previous doses using the data from two detailed specific activity *v.* time curves obtained previously. The largest correction was about 13% of the total ^{35}S in the fleece from the dose being studied.

In one comparison only (sheep MA51) the percentage of the dose incorporated into the wool was larger for the bound than for the free cystine. In each case the results for the free cystine were about the same even though the mass of cystine injected ranged from a fraction of a milligram to 2 g.

The initial rates of disappearance of the radioactivity from the circulating plasma after some of the doses of free and of disulphide-bound L-[^{35}S]cystine are shown in Figure 5. The results show that at least two-thirds of the free cystine disappeared from the blood stream in the first few minutes, irrespective of the mass of cystine injected. Thus, when the plasma–cystine mixtures were injected the free cystine was presumably removed in the first few minutes. Subsequently the rate of disappearance of the ^{35}S during the first 2 hr followed an exponential

function with a half-time of about 1 hr. This must approximately represent the disappearance of the disulphide-bound radioactivity since the amount of incorporation of cystine-^{35}S by protein synthesis during the 2-hr period was estimated to be no more than 20%.

TABLE 2

EFFICIENCY OF INCORPORATION OF ^{35}S INTO THE WOOL OF SHEEP AFTER INTRAVENOUS INJECTIONS OF L-[^{35}S]CYSTINE

Sheep No.	Dose			^{35}S in Fleece Grown during the First 4 Weeks after Each Dose (%)
	Form	Mass of Labelled Cystine (mg)	Amount of ^{35}S (μc)	
MA50	Cystine + plasma*	0·40	25·1	30
	Free cystine	0·64	40·3	28
	Free cystine	100	40·3	35
MA51	Free cystine	0·64	40·2	24
	Cystine + plasma*	0·32	20·0	37
	Free cystine	2000	40·3	24
MA52	Cystine + plasma*	0·43	27·3	28
	Free cystine	0·64	40·4	31
	Free cystine	500	40·2	30

* About 60% of the ^{35}S was in the form of half-cystine residues bound to the plasma proteins by disulphide bonds. See text for full description.

IV. DISCUSSION

The binding of cystine (or cysteine) and other compounds containing disulphide or sulphydryl groups to proteins is a well-known phenomenon which is especially important in the study of such compounds by the radioactive tracer technique (Lee *et al.* 1952; Tarver 1954; Samarina, Kritzman, and Konikova 1956; Eldjarn and Pihl 1956, 1957). Eagle, Oyama, and Piez (1960) attributed at least a part of the slow but continuing growth which was observed in mammalian cell cultures in a cyst(e)ine-free medium to the mobilization of bound cyst(e)ine. The marked stimulatory effect in this system of reduced inorganic sulphur compounds and of compounds such as D-cystine and glutathione was similarly attributed to the fact that they promote the release of the bound cystine residues from the serum proteins. They concluded that a reversible transfer of a half-cystine residue (CyS) from the cystine (CySSCy) to a protein thiol group (PrSH) occurred:

$$\mathrm{PrSH} + \mathrm{CySSCy} \leftrightharpoons \mathrm{PrSSCy} + \mathrm{CySH}. \qquad (1)$$

The present results (Figs. 1 and 2) agree with this and show that about two-thirds of a tracer amount of cystine is bound to the proteins of sheep plasma *in vitro* after

TABLE 3

SHOWING THE METHOD OF ESTIMATION OF THE PERCENTAGE OF DOSE OF ^{35}S IN THE FLEECE OF SHEEP MA51 AFTER TWO INTRAVENOUS INJECTIONS OF L-[^{35}S]CYSTINE

Dose	Clipped* Area	Growth Period (days)	Mass of Clean Dry Wool (g)	Specific Activity† (μc/g)	Total ^{35}S in Sample (μc)	Total ^{35}S in Fleece (μc)	% Dose in Fleece
40·2 μc free cystine (0·64 mg) injected on day 0	I	0–7	3·50	0·053	0·186	9·49‡	24
		7–14	4·32	0·131	0·567		
		14–21	4·05	0·027	0·109		
		21–28	4·21	0·015	0·062		
	Totals		16·1		0·924		
	II	0–7	2·85	0·042	0·120	9·15	
		7–14	3·59	0·130	0·465		
		14–21	3·37	0·029	0·099		
		21–28	3·37	0·014	0·048		
	Totals		13·2		0·732		
	Whole fleece	0–28	165	0·0582 0·0589 0·0621 0·0586		9·81	
	Means			0·0595		9·48 (9·5)§	
20·0 μc cystine +plasma injected on day 28	I	28–35	4·73	0·0457	0·216	8·18	37
		35–42	4·39	0·0792	0·348		
		42–56	10·64	0·0157	0·167		
		56–63	4·96	0·0093	0·046		
	Totals		24·7		0·777		
	II	28–35	4·00	0·0438	0·175	7·84	
		35–42	3·57	0·0756	0·270		
		42–56	9·02	0·0172	0·155		
		56–63	4·75	0·0088	0·042		
	Totals		21·3		0·642		
	Whole fleece	28–63	260	0·0347 0·0310 0·0347 0·0296		8·45	
	Means			0·0325		8·16 (7·14)§	

* The whole fleece was clipped just before each injection. Two tattooed areas (I and II), one on each side of the sheep, were usually clipped weekly.

† The sulphur content of the wool (3·02, 3·04, 3·02, 3·09; mean 3·04%) was determined by the method of Myers (1959). Other samples of the wool were oxidized by the method of Myers (1959) and the specific activity of the sulphur, as benzidine sulphate, was measured. The specific activity of the wool was then calculated.

‡ i.e. $0·924 \times 165/16·1 = 9·49$.

§ Values in parenthesis are mean values corrected for previous doses.

4 hr at 39°C. About the same proportion was bound whether DL- or L-[^{35}S]cystine was used, confirming that D-cystine can take part in the exchange. The binding occurred to some extent in the plasma from all the species examined, showing that the reaction is not a property of sheep plasma proteins only.

Eagle, Oyama, and Piez (1960) found that –S–S– and –S– compounds, including cystine, failed to effect significant dissociation of the bound ^{35}S, whereas the results here (Fig. 3) showed that the bound ^{35}S was displaced by the addition of more cystine. Assuming that human and sheep plasma proteins react in the same way with cystine, the probable explanation of these results is that different experimental conditions were used. Eagle, Oyama, and Piez used human serum which had been saturated with labelled cystine and dialysed before treatment with non-radioactive cystine. Under these conditions the concentration of thiols would have been negligible compared with that in normal serum, thus demonstrating that there is no exchange between cystine and the mixed disulphide, PrSSCy. In the present experiments, in which the sheep plasma was not altered except for the addition of small amounts of cystine, the exchange probably occurred by reversal of equation (1), since a small concentration of cysteine is probably all that is required. The cystine in normal plasma *in vitro* thus undergoes a continuous exchange between the free and the protein-bound forms.

Most of the published observations on thiol–disulphide exchanges have been made on systems *in vitro*, but it is probable that such exchanges occur *in vivo* as well. Jenson (1959) concluded that there are strong indications that such interchanges play a role in certain important physiological processes, including keratin production; and Eldjarn and Pihl (1957) stated: "Provided the reaction rates are relatively high, any variation in the ratio of total –SS– to total –SH effected by oxidation or reduction of any one component will necessarily affect the concentration of all other molecular species. This presumably is the situation in cells and tissues in which numerous thiols and disulphides may exist in a dynamic equilibrium". Apparently, however, no attempts have been made to demonstrate that such an equilibrium does exist *in vivo* or to study the rate of such exchanges under normal physiological conditions. The most reasonable interpretation of the results reported here is that L-[^{35}S]cystine rapidly equilibrates with the circulating free cyst(e)ine, that some of the [^{35}S]cyst(e)ine becomes bound to the plasma proteins *in vivo* (Fig. 4) as well as *in vitro* by disulphide exchange, and that this exchange occurs continuously with a half-time of about 1–2 hr in the circulating plasma (Figs. 4 and 5). Some of the ^{35}S presumably disappeared by equilibration of the injected plasma proteins with the extravascular pool but this is a relatively slow process, with a half-time of about 0·5–1 day, in other species (Sterling 1951; Cohen *et al.* 1956). The half-time of 1–2 hr in the present case is therefore mainly attributed to the thiol–disulphide exchange. The bound cystine has a much slower turnover rate than free cystine which has a half-time in plasma of only a few minutes at the most (Fig. 5). The recovery from the oxidized plasma proteins of most of the ^{35}S bound *in vivo* as cysteic acid shows that this ^{35}S was still in the form of half-cystine residues. The results in Figure 4 show that some binding occurs *in vivo*, but do not enable the concentration of bound cystine to be calculated.

Eagle, Oyama, and Piez (1960) did not discuss the possibility that some half-cystine residues are bound by the plasma proteins *in vivo* but from their data it may be calculated that the sera which they used may have contained disulphide-bound cystine *in vivo* at concentrations of at least 0·005–0·04 mM. This is based on three of their observations: (*a*) that freshly drawn serum or plasma contained free cyst(e)ine at

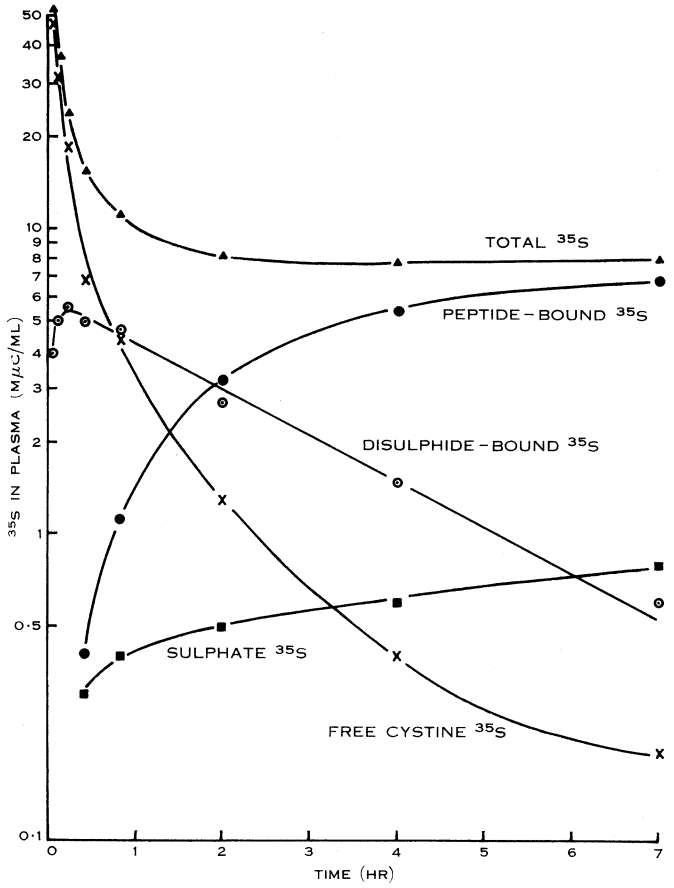

Fig. 4.—Distribution of ^{35}S in the plasma of a sheep after an intravenous dose of L-[^{35}S]cystine. L-[^{35}S]cystine (356 μc; 6·7 mg) was injected into a Corriedale ewe. Blood samples were taken at intervals, cooled immediately, and centrifuged at 5°C. The plasma was analysed within 1 hr after taking each blood sample to minimize the binding of [^{35}S]cystine by the proteins *in vitro*. The results show that disulphide binding occurs *in vivo*.

a concentration of 0·04–0·045 mM (as cystine); (*b*) that when horse or human serum, which had been stored at −20°C for 1–8 weeks and then dialysed, was heated to 100°C, a supernatant fluid containing 0·03–0·05 mM free cystine was obtained; and (*c*) that a maximum of 62% of the ^{35}S was released on heating plasma containing disulphide-bound [^{35}S]cystine at pH 7–9 to 100°C. From the second and third of these statements the total concentration of bound cystine in the aged serum must have been at least 0·05–0·08 mM, a concentration higher than that of the free cystine

in fresh serum by 0·005–0·04 mM. This conclusion must be regarded as being tentative only, because of the usually large experimental error of such determinations.

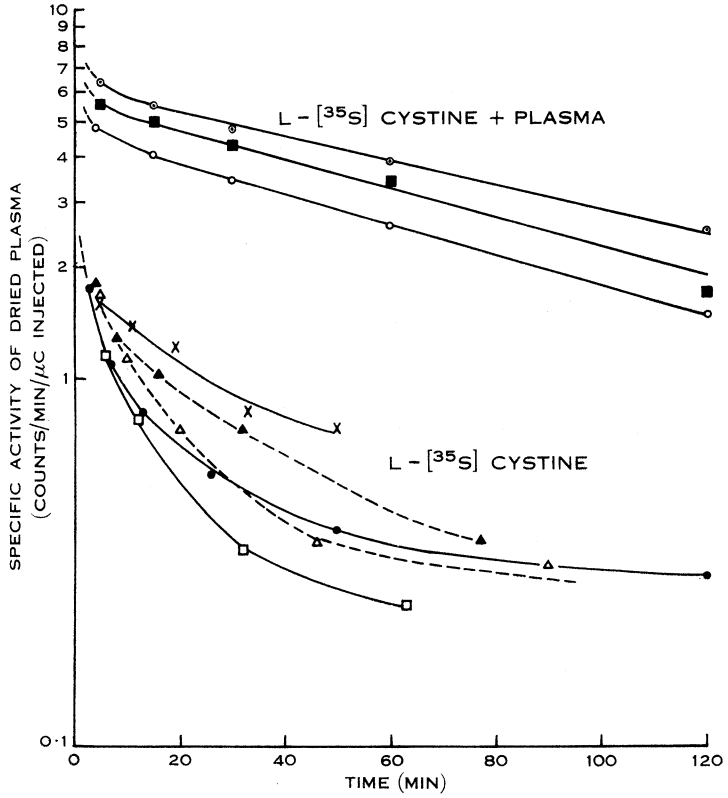

Fig. 5.—Rate of disappearance of ^{35}S from blood plasma after intravenous doses of L-[^{35}S]cystine, given as either free cystine or as cystine mixed with plasma long enough to ensure disulphide binding of 60% of the radioactivity by the plasma proteins. To compare the results more easily the counting rates of infinitely thick samples (1 cm^2) of dried plasma have been divided by the number of microcuries injected. Assuming instantaneous mixing of the dose with a plasma volume of 2 l. and a dry matter content of 7% the calculated zero point on the ordinate is about 7.

	Cystine + Plasma				Free Cystine		
	Sheep No.	Specific Activity (μc)	Cystine (mg)		Sheep No.	Specific Activity (μc)	Cystine (mg)
⊙	MA50	25·1	0·40	●	A459	356	6·7
■	MA51	20·0	0·32	□	MA50	40·3	0·64
○	MA52	27·3	0·43	△	MA50	40·3	100
				▲	MA52	40·2	500
				×	MA51	40·3	2000

Since half-cystine residues are apparently bound to the plasma proteins to some extent *in vivo* and since these residues are not removed by precipitation with picric acid, it is evident that the results obtained by the techniques of Stein and

Moore (1954) for the free cyst(e)ine levels in plasma do not represent the total amount of cyst(e)ine present in a relatively free form. The results also show that small and variable amounts of half-cystine residues are probably held by the plasma proteins isolated by fractional precipitation with either neutral salts or organic solvents. The amount of bound cystine would depend on the level of cyst(e)ine in the blood at the time of sampling, on the time that elapses before the fractionation is carried out, and on the temperature of the sample during this time. This could explain why dinitrophenyl ("DNP")-cysteic acid in addition to DNP-aspartic acid has been found in hydrolysates of DNP-equine serum albumin which had been oxidized with performic acid (Titani, Yoshikawa, and Satake 1956; Turner, Kennedy, and Haurowitz 1959) whereas DNP-aspartic acid alone has been detected in DNP-bovine and DNP-human serum albumin which had been similarly treated (Thompson 1958; Biserte 1959; Turner, Kennedy, and Haurowitz 1959; Ikenaka 1960).

In view of the fairly rapid exchange between free and bound cystine in plasma and the similarity in the percentage of the doses that appeared in the wool (Table 2) it is impossible to say from these results whether either of these forms of cystine play any special role in keratinization.

About 30% of each of the intravenous doses of L-[^{35}S]cystine was incorporated into the wool grown during the subsequent 4 weeks, whether the cystine was in the free or protein-bound form and irrespective of the mass of cystine injected, up to 2 g. This is interesting because 2 g is probably massive compared to the total pool of free and disulphide-bound cystine in the plasma and appears to be comparable with the amount of cystine required *per day* for protein synthesis in the whole animal.

There seem to be no figures for the concentration of free (or bound) cystine in sheep plasma but if we assume the level to be the same as in man, about 1·2 mg free cystine per 100 ml plasma (Stein and Moore 1954), there must be no more than about 3–4 mg of free plus disulphide-bound cystine per 100 ml plasma or a total pool of about 100 mg in the circulating plasma, that is assuming that the binding can occurr *in vivo* to the same extent as *in vitro*. The amount of cystine required per day was roughly estimated as follows. If we assume about 15% of the body weight of the sheep is protein, as quoted for other species by Siri (1956), and an average turnover time of 35 days (1·44 × half-time of 26 days (Downes 1961)), the amount of protein synthesized per day by the sheep used here (about 40 kg body weight) would be $(40 \times 0 \cdot 15 \times 1000)/35 = 170$ g per day. Taking 1·5 as the average percentage of cystine in the proteins of the entire animal (Block and Bolling 1951), about 2·5 g of cystine per day would be required. This is probably an upper limit because about one-third of the body protein is comprised of extracellular collagen which contains no cystine and is evidently turned over very slowly indeed (Neuberger and Slack 1953). The estimate of 2·5 g per day required for protein synthesis may be compared with an estimated 3·5 g of cystine supplied daily in the ration, as cystine plus methionine (1000 g ration per day; 10% protein containing 3·5% cystine, including methionine, and assuming it is all convertible to cystine). In addition an unknown amount of these amino acids may be synthesized by micro-organisms in the rumen. The sheep studied were producing about 6 g of clean dry

wool per day. In other words, about 600 mg of cystine per day was required for wool growth.

With such estimates in mind it was not unreasonable to expect that a large proportion of an intravenous dose of 2 g of cystine would be rapidly excreted. Therefore to explain the results (Table 2), either the rate of protein synthesis increased rapidly for a short period while the excess cystine was available or else the cystine was stored and used gradually over a longer period. The latter explanation, with a pool of at least several grams of cystine and with disulphide exchange as the mechanism, seems to be the more reasonable one. This explanation is consistent with the hypothesis that there is a metabolic pool containing cystine in the skin, perhaps in the wool follicles themselves (Downes 1961).

V. Acknowledgments

The author wishes to thank Dr. A. C. I. Warner for his helpful advice and for carrying out the bacteriological examinations; Dr. R. J. Walsh, Director, Blood Transfusion Service, Australian Red Cross Society, Sydney, for a sample of human plasma; and Messrs. L. F. Sharry and A. R. Till for their technical assistance.

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