and consequently, in the presence of protein the effective concentration of Cu is not known. We have re-examined the uptake of ^{64}Cu by erythrocytes from buffer and from plasma in order to establish the nature of the uptake process and to determine how it is modified in the presence of protein. Because of our interest in Cu metabolism of the sheep we have used sheep erythrocytes.

METHODS AND RESULTS

Erythrocyte suspension

Sheep blood cells were obtained from heparinised blood by centrifugation at 4 °C followed by repeated washing in 0.9% NaCl. If required, plasma was removed after the first centrifugation and kept at 0–4 °C until used. The washed, packed erythrocyte suspension was added to the incubation medium in the proportion of 40:60 which gave a haematocrit of about 35%, roughly equivalent to whole blood. All experiments were carried out on the day the blood was collected.

Incubation procedure

The incubations were carried out in a shaking water bath at 37 °C in conical flasks containing a total volume of 60–100 ml, from which 5-ml samples were taken. Uptake was stopped by addition of 5 ml of cold 0.9% NaCl, followed by centrifugation at 0–4 °C. The supernatant was removed and the cells washed a further three times with 5-ml quantities of cold 0.9% NaCl. The ^{64}Cu, and the stable Cu, if any, were generally added to the medium before addition of the erythrocyte suspension.

Media

The media in which the erythrocytes were suspended was either plasma or a solution containing buffered salts, *i.e.* Tyrode's solution or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (0.01 M) in a solution containing 8.48 g NaCl, 0.35 g KCl, 0.16 g KH_2PO_4, 0.34 g $MgCl_2 \cdot 6H_2O$, and 0.56 g $CaCl_2 \cdot 6H_2O$, per l adjusted to pH 7.2 with NaOH.

Except where otherwise stated, the concentration of Cu added to the medium was 100 μg/l, which is of the same order as the concentration of direct-reacting Cu found in plasma of normal sheep[4]. The specific activity of the added Cu was about 1.0 Ci/g in the plasma medium and about 0.25 Ci/g in the buffer.

Radioactivity measurements

$^{64}CuCl_2$ was obtained from the Radiochemical Centre, Amersham, with specific activity within the range 4–20 Ci/g Cu. Radioactivity determinations were carried out in a well-type automatic Gamma Spectrometer fitted with a 2-inch NaI crystal. Corrections in the counts for decay and, where necessary, the geometrical variation were made. After determination of radioactivity, samples were freeze-dried and ^{64}Cu uptake expressed in terms of weight of dry erythrocytes.

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Stable Cu analyses were carried out on a Hilger and Watt Atomic Absorption Spectrophotometer (Model AA2). Direct-reacting Cu in plasma was determined as described by Suttle and Field[5]. For total Cu, plasma was diluted with an equal volume

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of water before measurement. Packed cell volumes were determined by the standard micro-haematocrit technique. Glassware was soaked in Decon 75 (2%) solution overnight and rinsed in distilled deionised water.

Removal of ^{64}Cu in the washing procedure

The radioactivity taken up by the erythrocytes after incubation for 2.5 h was calculated from the difference between the total activity added and that remaining in the supernatant. Cells were washed 3 times and their activity measured. The difference between the two activities, $10.5 \pm 1.4\%$ (S.D.) for 12 replicates, was the amount of activity lost in the washings.

Comparison of buffered incubation media

The uptake of ^{64}Cu by sheep erythrocytes from HEPES, and Tyrode's solution (pH 7.2) containing Cu at concentrations of 63 and 630 μg/l was measured. The differences between the two media were small and not significant but since the uptake in HEPES was consistently slightly greater this was used in subsequent experiments.

The effect of gas phase

Erythrocyte suspensions in HEPES-buffered medium were incubated in an atmosphere of either air, a mixture of CO_2 (5%)/O_2 (95%), or N_2. No significant differences in the amount of ^{64}Cu taken up by erythrocytes after 2 h were apparent, and all subsequent experiments were therefore carried out in air.

Binding of ^{64}Cu to the cell walls

After incubation of erythrocytes with ^{64}Cu in HEPES buffer for 2 h the activity in the washed erythrocytes was determined in the normal way. After haemolysis by the addition of 20 vol. of 0.05 M Tris–HCl buffer, pH 8.0, the cell walls were separated by centrifugation at $3000 \times g$ for 10 min, washed three times with buffer and the radioactivity in the slightly pink pellet determined. The average activity remaining in the ghosts expressed as percentage of that present in intact erythrocytes was, for six replicates, 10.3 ± 0.35 (S.D.).

Effect of concentration of Cu in the media

Buffer. The uptake of ^{64}Cu was proportional to concentration from 0–2500 μg Cu/l, a range much greater than the normal range for direct-reacting Cu (Table I). The regression equation after 40 min incubation was: $y = 0.00461x - 0.141$ where y = uptake of Cu (μg/g dry erythrocytes) and x = concentration of added Cu (μg/l).

Plasma. Cu concentrations up to 900 μg/l plasma were used. The uptake by erythrocytes was proportional to concentration of added Cu (Table II). The regression equation, for the same erythrocytes as used in buffer, after 45 min incubation was $y = 0.000641x - 0.0401$. For a given concentration of added Cu in the medium the uptake from plasma was much less than from buffer and was also slower in reaching equilibrium.

Kinetics of uptake process

From the measurement of the rate of Cu uptake at different concentrations it is possible to determine the order of the reaction, *i.e.* the number of chemical species

TABLE I

UPTAKE OF Cu FROM BUFFER BY SHEEP ERYTHROCYTES

33 ml packed erythrocyte suspension = 9.34 g dry wt added to 50 ml buffer. 5 μCi ^{64}Cu containing 0.15 μg Cu added.

Time of incubation (min)	*μg Cu uptake/g erythrocytes*		
	Concentration of added Cu (μg/l):		
	100	*500*	*2500*
11	0.294	1.64	9.35
15	0.312	1.76	10.00
25	0.342	1.85	10.75
40	0.390	2.08	11.40
50	0.440	2.35	12.00

TABLE II

UPTAKE OF Cu FROM PLASMA BY SHEEP ERYTHROCYTES

33 ml packed erythrocyte suspension = 9.34 g dry wt added to 50 ml plasma. 5 μCi ^{64}Cu containing 0.15 μg Cu added.

Time of incubation (min)	*μg Cu uptake/g erythrocytes*			
	Concentration of added Cu (μg/l):			
	100	*300*	*600*	*900*
11	0.016	0.051	0.110	0.192
34	0.035	0.113	0.248	0.432
45	0.042	0.141	0.318	0.556
65	0.045	0.162	0.350	0.634
100	0.047	0.167	0.377	0.659

whose concentrations determine the velocity of the process, from the expression:

$$n = \frac{\log(-dc_1/dt) - \log(-dc_2/dt)}{\log C_1 - \log C_2}$$

where n = order of reaction, $-dc_1/dt$ and $-dc_2/dt$ are the rates of decrease in Cu concentrations C_1 and C_2. An estimate of dc/dt can be obtained by determining $\Delta c/\Delta t$ for an appreciable time interval. The means of the concentrations at these intervals are then used for values of C. Application of this calculation to data shown in Tables I and II indicate that the reaction for both buffer and plasma is of the first order. For example, taking values for Cu concentrations of 100 and 500 μg/l after 25 min incubation in buffer, a value for n of 1.07 is obtained. The corresponding value using Cu concentrations of 500 and 2500 μg/l and 15 min incubation is 1.12. Similar calculations for plasma after 34 min incubation at Cu concentrations of 100 and 300 μg/l give $n = 1.02$ and at 45 min and Cu concentrations 600 and 900 μg/l,

$n = 1.22$. These results indicate that although rates of uptake differ markedly in plasma and buffer the basic process is similar.

The velocity constant (k) for a first order reaction can be calculated from the expression: $k = (2.303/t) \log (a/a-x)$ where a = initial concentration of reactant and x = decrease in concentration after time t. The plot of log $(a-x)$ against t is a straight line with a slope $= -2.303/k$. Using the results for the buffer experiment in Table I, where the effective initial Cu concentration (a) is known, values for k for the three concentrations 100, 500 and 2500 μg Cu/l were 0.0175, 0.0196 and 0.0181, respectively, giving a mean value of 0.0184 min^{-1}. Since the velocity constant is fixed for a given temperature this value for k can be used to calculate the value of a for plasma from the data in Table II. The values ranged from 10.5 to 21.6% of the added Cu, the mean values being 13.5, 15.0, 16.1 and 19.3% for added Cu concentrations of 100, 300, 600 and 900 μg/l, respectively. The increase in the values with concentration of added Cu was significant ($P < 0.01$).

Effect of percentage plasma in the medium

Fig. 1 shows results of an experiment in which erythrocytes were incubated in different mixtures of buffer and plasma to which ^{64}Cu was added. The buffer contained 100 μg/l added Cu as $CuCl_2$ and the plasma had a direct-reacting Cu concentration of 63 μg/l. Where no plasma was present the uptake had reached a maximum value at 1 h whereas in the presence of plasma it was still continuing at 2 h. The

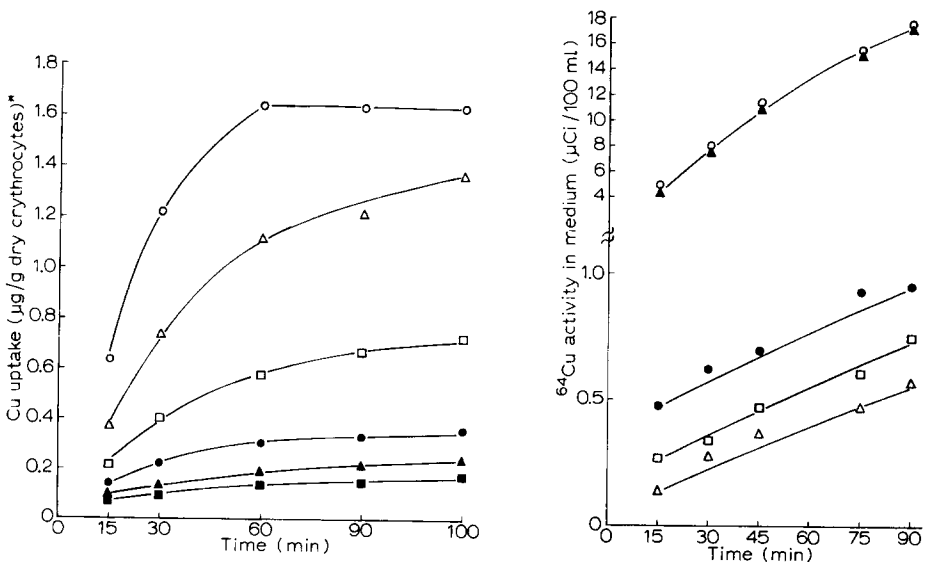

Fig. 1. Uptake of Cu by erythrocytes from buffer/plasma mixtures. ○—○, buffer; △—△, buffer–plasma (9:1, v/v); □—□, buffer–plasma (3:1, v/v); ●—●, buffer–plasma (1:1, v/v); ▲—▲, buffer–plasma (1:3, v/v); ■—■, plasma. * Calculation assumes mixing of added Cu with plasma direct-reacting Cu (63 μg/l).

Fig. 2. Effiux of Cu from labelled erythrocytes incubated in plasma and buffer with and without added Cu. Erythrocytes were incubated in buffer + ^{64}Cu containing 100 μg Cu per l for 1 h and washed before resuspension. ○—○, plasma without added Cu; ▲—▲, plasma + 100 μg Cu/l; ●—●, buffer without added Cu; □—□, buffer + 100 μg Cu/l; △—△, buffer + 200 μg Cu/l.

reduction in ^{64}Cu uptake due to plasma was not proportional to the concentration of plasma in the medium; a small amount of plasma resulted in a large reduction in uptake and succeeding increments in plasma concentration had diminishing effects.

Efflux of ^{64}Cu from labelled erythrocytes

Erythrocytes were labelled with ^{64}Cu by incubation for 1 h in HEPES buffer. The cells were then washed and resuspended in various media in the normal manner but the reaction was stopped by taking samples (3 ml) into normal saline (8 ml) at −2 °C. Fig. 2 shows a comparison of the efflux from labelled erythrocytes which had been suspended in buffer and plasma with and without added Cu. In buffer, but not in plasma, the presence of Cu in the medium inhibited the efflux of ^{64}Cu from the cells. The efflux into plasma was much greater than into buffer. In order to establish whether the effect of plasma was due to the ability of plasma protein to bind Cu, or to amino acids facilitating transport, a second experiment was conducted in which cells were resuspended in buffer, buffer + histidine ($0.75 \cdot 10^{-4}$ M), buffer + GSH ($1 \cdot 10^{-3}$ M), whole plasma, plasma ultrafiltrate prepared by filtration through a PM 30 Diaflo Ultrafilter (Amicon, High Wycombe) and the large-molecular weight fraction from the same ultrafiltration diluted to its original volume with buffer. No Cu was added to the media. In their effects on efflux the media fell into three clearly defined groups. Efflux was greatest in whole plasma and the plasma protein fraction, intermediate in plasma ultrafiltrate and buffer + GSH either with or without histidine, and least in buffer and buffer + histidine. There were no significant differences between the members of each group, and after 90 min incubation the relative mean concentrations of Cu released into the media were, for the three groups, 12:5:1.

Effect of inhibitors

EDTA (1 mM), iodoacetate (2.5 and 5 mM), NaF (2.5 and 5 mM) and 2,4-dinitrophenol (1 mM), caused no reduction in Cu uptake from buffer. However, *p*-chloromercuribenzoate (1 mM) and GSH (1 mM) both reduced Cu influx, although after 2 h incubation the effect of the former was no longer apparent. Expressed as percentage of controls, values after 0.5 h were 62 and 43, and after 2 h, 107 and 77 for *p*-chloromercuribenzoate and GSH, respectively. Subsequently, the effect of GSH on uptake of ^{64}Cu from plasma was examined. GSH (1 mM) was added (a) to plasma immediately before addition of ^{64}Cu and (b) to ^{64}Cu before addition to the plasma. A marked reduction in uptake compared with controls was apparent and was similar for both (a) and (b), mean values being 30% and 22% at 0.5 h and 2 h, respectively. The increasing percentage inhibition with time of incubation was due to a continuing more rapid uptake in the control.

The effect of histidine on uptake of ^{64}Cu from plasma and from buffer

Addition of histidine in approximately physiological concentration ($0.75 \cdot 10^{-4}$ M) and 10 times this concentration to dialysed plasma caused only a small increase in ^{64}Cu uptake with histidine concentration (Table III). On the other hand the uptake of histidine was proportional to its concentration, so that the molar ratios of histidine: Cu in the cell were also proportional to histidine concentration. The effects of adding L-histidine, in the same concentrations as used in the plasma experiment, to buffer containing 100 μg Cu per l are also shown in Table III. There was no increase in

TABLE III

THE EFFECT OF HISTIDINE CONCENTRATION ON THE UPTAKE OF ^{64}Cu AND HISTIDINE BY ERYTHROCYTES FROM BUFFER AND THE NON-DIFFUSIBLE FRACTION OF DIALYSED PLASMA

33 ml packed erythrocytes added to 50 ml plasma dialysed against 3 × 30 vol. of 0.9% NaCl. ^{64}Cu (100 μg Cu/l) and L-[2,5-^{3}H]histidine (spec. act. 4 μCi/mg) added. After decay of ^{64}Cu and haemolysis with 2 vol. water samples were prepared for scintillation counting by the method of Kumar and Berger[15].

	Uptake (μg/ml packed erythrocytes) and molar ratio Cu: His at stated times of incubation								
	1 h			*1.5 h*			*2 h*		
	Cu	*His*	*Cu/His*	*Cu*	*His*	*Cu/His*	*Cu*	*His*	*Cu/His*
Buffer + Cu	0.23	—	—	0.23	—	—	0.29	—	—
Buffer + Cu + histidine (15 mg/l)	0.22	1.14	0.49	0.22	1.35	0.41	0.27	1.96	0.34
Buffer + Cu + histidine (150 mg/l)	0.23	11.6	0.050	0.21	14.7	0.035	0.27	20.9	0.031
Plasma non-diffusible fraction + Cu	0.049	—	—	0.053	—	—	0.072	—	—
Plasma non-diffusible fraction + Cu + histidine (15 mg/l)	0.053	0.75	0.18	0.055	1.08	0.13	0.077	2.15	0.089
Plasma non-diffusible fraction + Cu + histidine (150 mg/l)	0.061	10.7	0.014	0.064	12.9	0.012	0.087	16.5	0.013

the ^{64}Cu uptake due to the addition of histidine at either concentration whereas histidine uptake was again proportional to its concentration.

DISCUSSION

The validity of using the radioactivity of washed erythrocytes as a measure of the Cu uptake depends on establishing that the activity is predominantly within the cells and not associated with the cell membrane, and also that the procedures used to wash cells free of suspending medium do not remove a large proportion of the activity originally present. It is known[6,7] that Cu exists in at least two compartments within erythrocytes. However, the proportion of the total which could be due to cell wall binding has not been determined. The finding that approximately 10% of the Cu was bound to the cell wall may well be a high estimate, since the ghosts had a pink appearance after washing, indicating the presence of some cell contents. The loss of activity during the washing procedure was also approximately 10%.

The calculation of the order of the reaction from the uptake at different concentrations in both buffer and plasma shows that the reaction is of the first order in both media. If the process were facilitated diffusion, which involves the participation of a carrier in the membrane[8], pseudo-first order kinetics could operate only at concentrations of Cu at which the concentration of the carrier was in excess. However, no evidence of carrier saturation was observed in our buffer experiments where concentrations of Cu were up to 25 times the normal plasma direct-reacting Cu. Assuming that the effective concentration is only about 13% of the Cu estimated as direct reacting then concentrations in buffer were up to 150 fold those existing in normal plasma. It is likely therefore that if a carrier were involved evidence of its saturation would have been obtained. From these results together with the inhibition studies, which indicate that active transport is not involved, we conclude that the process is simple diffusion as was stated by Saltman *et al.*[1] for Cu uptake by tissue slices. A similar mechanism has been shown to exist for uptake of Mn by erythrocytes[9].

The large difference in rate of uptake in plasma compared with buffer does not indicate a fundamental difference in the process involved, but can be accounted for by the ability of the plasma proteins to bind Cu thus effectively reducing its concentration. This was demonstrated in the experiments in which buffer and plasma were mixed in different proportions, and also in the efflux experiments. This interpretation agrees with the calculated effective concentration of Cu in plasma which indicates that, at concentrations of Cu similar to those existing *in vivo*, only approximately 13% of the added Cu is "available" to the erythrocytes. This figure is very close to that of 16.5% determined by Lau and Sarkar[10] in studies with purified albumin for the relative concentration of Cu in the Cu–(histidine)$_2$ complex existing in the equilibrium mixture of Cu, albumin and histidine. The formation of such a complex, and its possible role in facilitating removal of Cu from albumin in the form of Cu–(histidine)$_2$ complex, offers an explanation for the effect of adding histidine to dialysed plasma in increasing Cu uptake, which was reported by Neumann and Silverberg[3] and has been confirmed by us. However, it is not known whether the Cu enters the erythrocyte coupled to histidine or whether the Cu–(histidine)$_2$ complex can be broken down to allow the Cu to enter the erythrocyte alone. Our studies

with histidine do not resolve this question since it is known that free histidine can be taken up by the erythrocyte[11]. Certainly histidine is not essential to the process of uptake since in the absence of plasma protein, *i.e.* in buffer, histidine has no effect on the rate.

The finding that the proportion of the added Cu which is available to the erythrocytes increases with the amount of Cu in the medium suggests that as concentrations increase the Cu may occupy different binding sites on the albumin molecule. It has been shown[12,13] in studies with purified Cu-free albumin that Cu can bind to more than one site and that the strength of binding decreases above a molar ratio of Cu:albumin of 1. The preferred binding site involves the N-terminal amino acid. However, assuming a molecular weight of 68000 for albumin and a plasma albumin concentration of 31 g/l (ref. 14), it can be calculated that, for concentrations of direct-reacting Cu of the order which exist *in vivo*, less than 1% of the albumin molecules have Cu bound to them. Therefore, if added Cu occupies different sites the preferred Cu-binding site must be occupied in many cases by other chemical species.

The inhibitory effect of GSH may be due to the formation of a Cu–GSH complex in the medium. Evidence for such a complex comes from an observed change in absorption spectrum on the addition of $CuCl_2$ to GSH (Smith, B. S. W. and Wright, H., unpublished). Since the red cell membrane is impermeable to GSH[11] it is likely that it is also impermeable to the Cu complex. The fact that the presence of GSH in buffer increased the efflux from labelled cells supports this interpretation of its mode of action. The action of *p*-chloromercuribenzoate was less marked and only apparent for the first 2 h, and may be due to steric hindrance following combination with SH groups on the cell membrane, rather than to inhibition through combination with enzyme groups.

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