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Binding and Clearance of Radioactive Adrenaline and Noradrenaline in Sheep Blood

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ABSTRACT

An understanding of the conditions influencing protein binding of catecholamines (CAs) is important in studying their metabolic effects. Unfortunately, reports on plasma protein binding of CAs are scarce, conflicting and mainly performed *in vitro*. The aim of our *in vivo* and *in vitro* studies was to investigate binding and clearance of radioactive adrenaline (epinephrine) (^{3}H-A), noradrenaline (norepinephrine) (^{3}H-NA) and their metabolites in sheep blood. The time course of the radioactivity in the blood after intravenous injection of ^{3}H-A and ^{3}H-NA (3.7 MBq each) in 4 sheep (2 of each sex; total of 8 administrations) was determined. Blood samples were taken from the jugular vein. The highest radioactivity was observed in the first sample (5 min) following injection. Radioactivity showed a biphasic disappearance. An initial stage, in which radioactivity decreased rapidly (within 1 h) after the injection, was followed by a slow stage, lasting for up to 1 month, until background levels were reached. *In vitro* results indicated that NA and A were present not only in plasma (70%) but also in the erythrocytes (30%; mainly bound to haemoglobin). Sephadex G-25 gel filtration revealed that from the plasma fraction about 15% was strongly bound to proteins (mainly albumin). These results demonstrate that previous experiments in this field have overestimated the percentage of CAs bound to plasma proteins, because binding to haemoglobin was previously not known. In the future, efforts should be made to characterize the adduct products of CAs and establish an assay to determine them *in vivo*. If this could be achieved, it would yield a valuable tool for measuring the stress experienced for a longer period.

Keywords: catecholamines, epinephrine, haemoglobin, plasma protein binding, ruminants

Abbreviations: A, adrenaline; BSA, bovine serum albumin; CA, catecholamine; NA, noradrenaline

INTRODUCTION

It is well known that many hormones circulating in the blood are bound to proteins. Several authors found such a binding of CA or their derivatives. They used various techniques, such as equilibrium dialysis (Powis, 1975; Sager *et al.*, 1987; Boomsma *et al.*, 1991; Trovik *et al.*, 1992), electrophoresis (Mirkin *et al.*, 1966; Teixeira *et al.*, 1979) and gel filtration (Zia *et al.*, 1971; Danon and Sapira, 1972; May *et al.*, 1974; Powis,

1975; Teixeira *et al.*, 1979). Although some authors could not find any interaction between CA and albumin (Bickel and Bovet, 1962; Mirkin *et al.*, 1966), others reported the binding of A and NA to serum albumin (Danon and Sapira, 1972), α- or β-globulins (Mirkin *et al.*, 1966; Teixeira *et al.*, 1979), α-acid glycoprotein (Sager *et al.*, 1987), lipoproteins and immunoglobulin M (Powis, 1975). It was concluded that the binding of A involves the alkyl side chain (Zia *et al.*, 1971; Teixeira *et al.*, 1979) or the phenolic hydroxy groups (Danon and Sapira, 1972). May and colleagues (1974) provided further evidence that NA and some of its derivatives bind to plasma proteins with the phenolic hydroxy groups, as well as the alkyl side chain. Boomsma and colleagues (1991) suggested that the specific binding of NA to human serum albumin is due to the presence of one or more oxidation products of NA. It has been suggested that the interaction of CA and proteins is of an irreversible nature (Teixeira *et al.*, 1979). The aim of our *in vivo* and *in vitro* studies with radiolabelled A and NA was to investigate binding and clearance of CA in the blood of sheep.

MATERIAL AND METHODS

In vivo experiment

Animals. Four mature healthy sheep (two of each sex) from the research farm of the University of Veterinary Medicine, Vienna were used (permission for the animal experiment: GZ 68.205/25-Pr/4/2000). The sheep (mixed breed, 50–60 kg) were 12 years old and were kept at the Clinic of Ruminants (El-Bahr *et al.*, 2005).

Administration of ^{3}H-CAs. ^{3}H-A or ^{3}H-NA was administered intravenously to two ewes and two rams (total of 8 administrations). ^{3}H-A (special synthesis, WS03DC, epinephrine, *levo*-ring-2,5,6-^{3}H) and ^{3}H-NA (NET 678, norepinephrine, *levo*-ring-2,5,6-^{3}H) were obtained from New England Nuclear (Boston, MA, USA). Both had a specific activity of 40–80 Ci/mmol (purity $>97\%$). The ^{3}H-CAs (3.7 MBq = 0.1 mCi) were administered in 10 ml sterile 0.9% NaCl solution. To mimic physiological conditions, the injection lasted for 1 min (approximately 6 ng A or NA per second) using canulation (Vasocan-Braunüle, 0.8 mm × 25 mm, Melsungen, Germany) of the jugular vein.

Collection of the samples. Blood was collected before (background level) and 5, 10, 30 and 60 min after the injection and then after 24, 48, 72 and 96 h and after 1, 2, 3 and 4 weeks. In the ewes, samples were taken only for 3 days and in the rams not all samples were collected during the first 24 h. Blood was taken from the jugular vein, using sterile disposable needles (1.5 mm × 50 mm) into heparinized tubes and the tubes were centrifuged at 3000*g* for 15 min. Plasma was stored at –24°C.

Measurement of radioactivity and calculations. Duplicates of plasma samples (0.5 ml) were mixed with 6 ml of scintillation fluid (Quicksafe A, no. 1008000, Zinsser Analytic, Maidenhead, UK) and measured in a liquid scintillation counter (Packard Tricarb 4640, Warrenville, IL, USA). Radioactivity was expressed as disintegrations per second (Bq) per litre of blood plasma.

In vitro experiments

Binding of ^{3}H-CAs to the blood cell and the plasma fraction. Blood samples were taken from 5 female sheep (2 years old) from the jugular vein into heparinized tubes. ^{3}H-NA or ^{3}H-A (1 μCi was used for all *in vitro* incubations) were incubated for 1 h with 1 ml whole blood (in duplicates) in reaction vials (1.5 ml) on a shaker (Thermomixer; stage 10, Eppendorf, Germany) at 38°C. Following incubation, the vials were centrifuged (14 000*g*; 15 min), the supernatant was transferred into scintillation vials, and the radioactivity was measured. In addition, ^{3}H-NA or ^{3}H-A was added to 1 ml plasma (in duplicates) from the same sheep. Following incubation (1 h), Sephadex G-25 (30×1.5 cm; Pharmacia Biotech AB, Uppsala, Sweden) gel filtration of the plasma samples was carried out in 0.1 mol/L sodium phosphate buffer. ^{3}H-NA or ^{3}H-A added to 1 ml distilled water served as control. A total of 36 fractions (2 ml each) were collected and the radioactivity was measured. The protein concentration in each fraction was determined spectrophotometrically at 280 nm. Protein-containing fractions were pooled. For investigation of which group of proteins the CAs bind, the protein fractions were incubated with ammonium sulphate (55%; which was determined to be the best concentration to precipitate the globulins, leaving the albumin in solution) overnight at 4°C on an orbital shaker. After centrifugation (3000*g*; 15 min), precipitated proteins were redissolved in phosphate buffer (0.1 mol/L; pH = 7.2). All samples were dialysed against the same buffer for 12 h at 4°C (dialysis buffer was changed three times). Afterwards, the radioactivity was determined in the supernatant and the precipitated protein fraction.

For determination of binding sites within the erythrocytes, whole blood samples (n = 2) were collected from 2 ewes. After centrifugation (3000*g*; 15 min), erythrocytes were harvested and washed twice with 5 ml sterile 0.9% NaCl solution. ^{3}H-NA or ^{3}H-A was added and incubated for 1 h. Afterwards the erythrocytes were washed three times and haemolysed by deep freezing. Haemoglobin (0.5 ml) was transferred to the Sephadex G-25 column as described above. To avoid quenching, only 10 μl of each fraction was used for counting the radioactivity. Haemoglobin concentration in each fraction was determined spectrophotometrically at 400–440 nm.

In addition, heparinized blood samples of one *in vivo* administration (^{3}H-NA) of day 1 and day 7 after the injection, respectively, were chosen to determine the possible binding of CAs to plasma proteins. After centrifugation, 1 ml of plasma of each sample was transferred to the Sephadex G-25 column and processed as described above.

Binding of ^{3}H-NA to plasma proteins (acetone precipitation). In reaction vials (1.5 ml) ^{3}H-NA (0.1 ml) was added to 0.7 ml of sheep plasma and 0.1 ml ascorbic acid (56.8 mmol/L) as an antioxidant as described by Boomsma and colleagues (1991). After incubation for 1 h, unlabelled NA (0.1 ml) was added separately in amounts of 100 μg, 1 μg, 100 ng, 10 ng and 100 pg, respectively (n = 5). After a further incubation period of 1 h, plasma proteins were precipitated by the addition of acetone (100 μl) and separated by centrifugation (14 000*g*; 15 min). Radioactivity was counted in both the supernatant and the precipitate. The experiment was repeated with changed order of adding labelled and unlabelled NA.

Type of binding between ^{3}H-CAs and albumin. For determination of the type of binding of CAs to albumin, 0.1 ml ^{3}H-NA or ^{3}H-A (n = 10) was incubated (1 h) with 0.8 ml BSA (40 mg/ml phosphate buffer) and 0.1 ml ascorbic acid (56.8 mmol/L). Following incubation, 1 ml of each mixture was separated on the Sephadex G-25 as described above. Each albumin-containing fraction was divided into two aliquots. Trichloroacetic acid (0.5 ml; 1.2 mol/L) was added to one aliquot to denature the proteins. Thereafter, the vial was shaken (5 min) and centrifuged (14 000g; 5 min). The radioactivity of the supernatant (0.5 ml) and of the untreated aliquot was measured in the liquid scintillation counter.

RESULTS

Radioactivity in the blood

In vivo, the highest radioactivity (65–279 kBq/L) in the blood was observed in the first sample (5 min) following injection. Radioactivity showed a biphasic disappearance. An initial stage (within 1 h), in which radioactivity decreased rapidly after the injection, was followed by a slow stage, lasting for 1 month, until background levels were reached. Representative graphs of injections of each hormone in the blood of an ewe and a ram are given in Figure 1.

Binding of ^{3}H-CAs to the blood cell and the plasma fraction

In vitro results indicated that NA and A were present not only in plasma (NA, 71.4% ± 3.5%; A, 70% ± 4.4%) but also in the erythrocytes (NA, 28.6% ± 3.5%; A, 30.0% ± 4.4%). After Sephadex G-25 filtration of ^{3}H-NA and ^{3}H-A (control samples), one peak of radioactivity, eluting around fraction 12, was detected (Figure 2a). After filtration of plasma samples of ewes that had been incubated with ^{3}H-NA and ^{3}H-A (Figure 2b), or of plasma samples of the *in vivo* radiometabolism study (^{3}H-NA) of day 1 and day 7 (Figure 2d), two radioactive peaks, eluting in the fractions 5 and 12 were detected. Proteins were present in fractions 4, 5 and 6 (highest concentration in fraction 5) as ascertained by spectrophotometric measurement. Sephadex G-25 gel filtration showed that 15.3% ± 0.9% (NA) and 14.9% ± 0.5% (A) of the radioactivity in the plasma was bound to proteins (Table I). From this portion, 70.1% ± 3.4% (NA) and 69.6% ± 6.2% (A) was found to be bound to albumin by precipitation with ammonium sulphate (Table I). Gel filtration of haemoglobin of the erythrocytes that had been incubated with ^{3}H-NA and ^{3}H-A revealed one radioactive peak eluting in fractions 4–6 (Figure 2c). Haemoglobin was present in the same fractions.

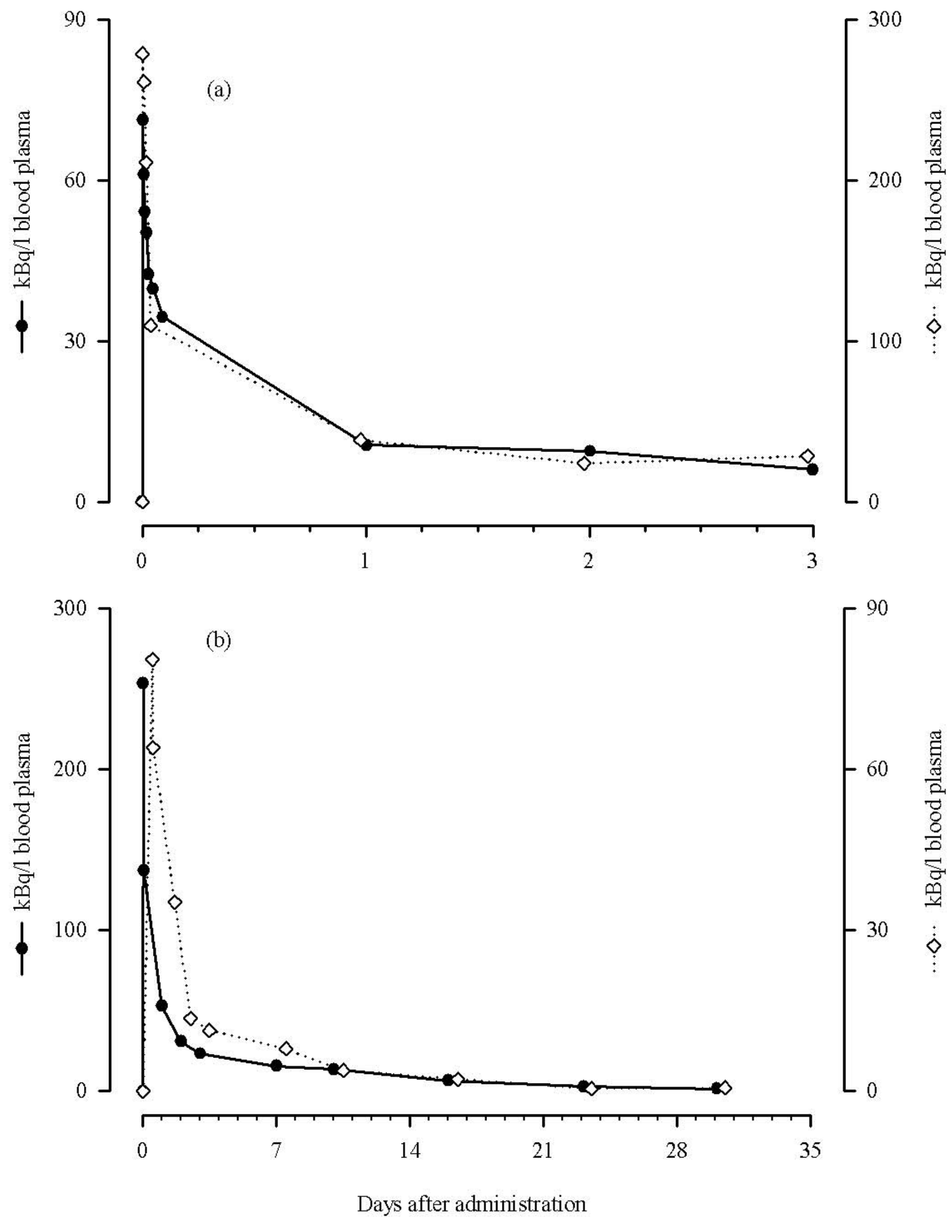

Figure 1. Radioactivity after injection of [^{3}H]noradrenaline (●) and [^{3}H]adrenaline (◇) in the blood of a ewe (a) and a ram (b); note the different scale of the Y axis

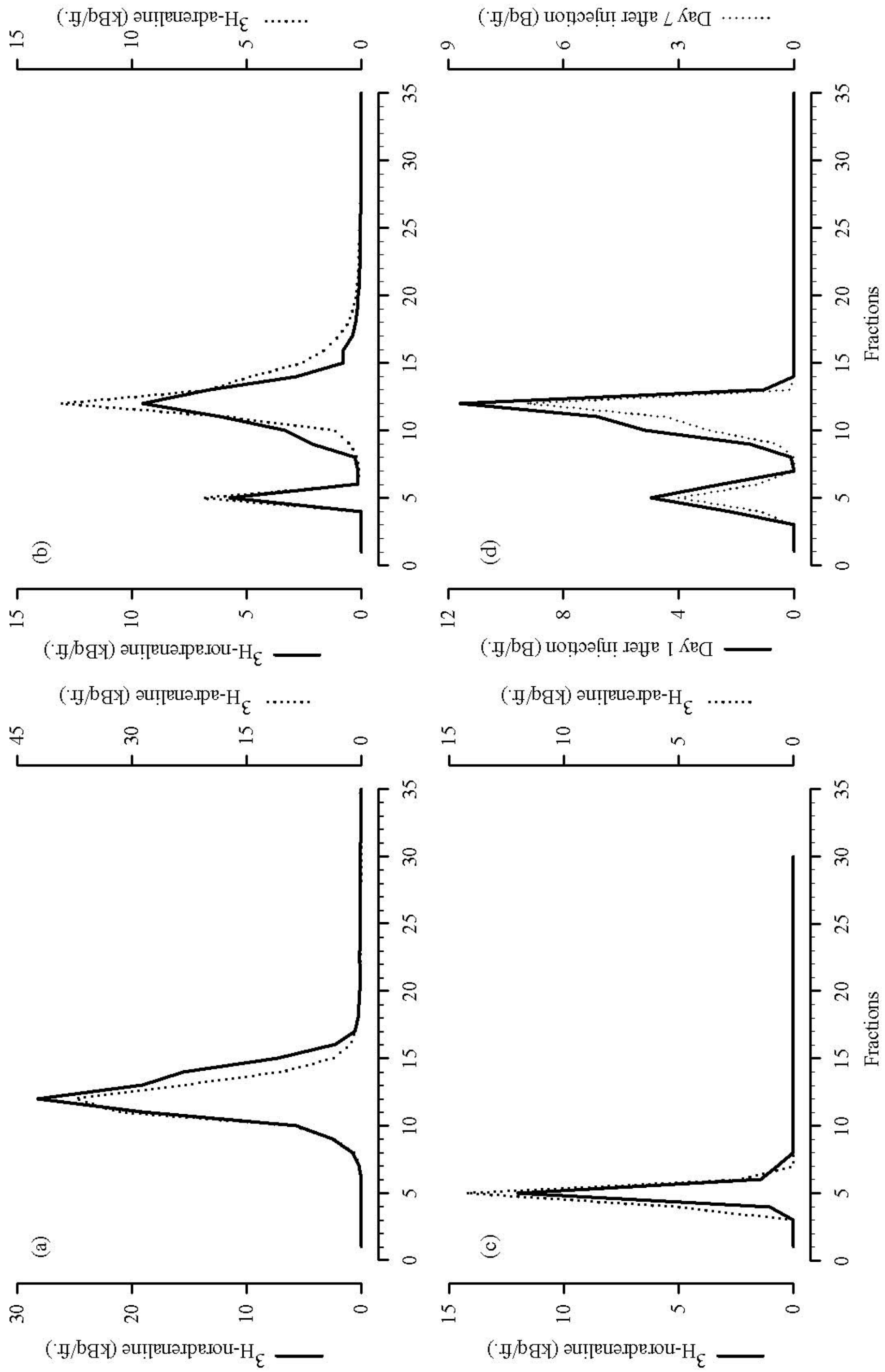
(a)
(b)
(c)
(d)
3H-noradrenaline (kBq/fr.)
3H-adrenaline (kBq/fr.)
Day 1 after injection (Bq/fr.)
Day 7 after injection (Bq/fr.)
Fractions
Fractions

TABLE I
Percentage (mean ± standard deviation) of binding of [^{3}H]adrenaline and [^{3}H]noradrenaline to the blood cell and the plasma fraction (*in vitro*)

Parameter	[^{3}H]Noradrenaline	[^{3}H]Adrenaline
CA in erythrocytes	28.6% ± 3.5%	30.0% ± 4.4%
CA in plasma fraction	71.4% ± 3.5%	70.0% ± 4.4%
Radioactivity in protein-free fraction	84.7% ± 0.9%	85.1% ± 0.5%
Radioactivity in plasma protein fraction	15.3% ± 0.9%	14.9% ± 0.5%
CA bound to albumin	70.1% ± 3.4%	69.6% ± 6.2%
CA bound to globulin	29.9% ± 3.4%	30.4% ± 6.2%

Binding of ^{3}H-NA to plasma proteins (acetone precipitation)

The percentage of binding of ^{3}H-NA to plasma proteins was not affected by the different concentrations of NA used or by the order of adding labelled and unlabelled NA (*t*-test, $p = 0.074$). When ^{3}H-NA was added first, the sum of radioactive substances recovered from the supernatant and the protein precipitate accounted for 90% ± 0.3% (mean ± SD) of the radioactivity added. From this, 11.5% ± 0.7% was present in the protein fraction. When unlabelled NA was incubated first, the radioactivity recovered from the supernatant and the protein precipitate accounted for 92% ± 0.5%. From this, 12.6% ± 0.5% was present in the protein fraction.

Type of binding between ^{3}H-CAs and albumin

When the amounts of radioactivity present in the controls were compared with the acid-treated portions, 94% ± 0.5% of CAs were precipitated together with albumin. This percentage was the same for both CAs (*t*-test, $p = 0.960$).

Figure 2 (opposite). Sephadex G-25 separation of [^{3}H]noradrenaline (—) and [^{3}H]adrenaline (......) incubated with distilled water (a), ewe's plasma (b) or haemoglobin (c), and plasma samples of sheep VI at day 1 (—) and day 7 (......) after the injection of [^{3}H]noradrenaline (d)

DISCUSSION

An understanding of the conditions influencing protein binding of CAs is important in studying their metabolic effects. Unfortunately, reports on serum protein binding of CAs are scarce, conflicting and mainly performed *in vitro*. The aim of our *in vivo* and *in vitro* studies was to investigate binding and clearance of ^{3}H-A, ^{3}H-NA or their metabolites in the blood of sheep. Blood was sampled within short intervals immediately after the injection and for only 3 days in the earlier-performed administrations (ewes), as others have reported a short half-life for CAs (Bühler *et al.*, 1977; Granner, 1996). In the experiment in rams, the blood samples were collected within longer intervals during the first day after injection, but afterwards up to one month, to monitor the return to background levels.

In the blood, radioactivity showed a biphasic disappearance after the administration of ^{3}H-A and ^{3}H-NA. In the first phase, the radioactivity decreased rapidly (within 1 h), indicating a short biological half-life of CAs. This is in agreement with former reports (Axelrod *et al.*, 1959; Bühler *et al.*, 1977; Granner, 1996). In the second phase, the radioactivity in the plasma decreased slowly until background levels were reached (after 1 month). The reason for the long half-life of the radioactivity in sheep may be an intravascular storage mechanism due to a strong binding of CA to plasma proteins, which protects some of the circulating CAs from elimination. This would be in agreement with the fact that in sheep the half-life of plasma proteins is of about 2 weeks (Kaneko, 1997), in contrast to a few minutes for the free CA. To investigate the type of binding of CA in the blood, samples were chosen from one ram at day 1 and day 7 after the injection of ^{3}H-NA. There was not enough radioactivity present in the plasma to determine the binding to albumin and globulin separately. The only information derived from this experiment was that NA was bound to plasma proteins. Consequently, some *in vitro* experiments were performed to investigate the binding systems of CAs in the blood in more detail (qualitatively and quantitatively).

In all *in vitro* experiments, CAs were incubated with whole blood, plasma or serum albumin for 1 h, because maximum binding was reported to occur in that incubation period (Boomsma *et al.*, 1991). *In vitro* results of whole-blood incubations indicated that NA and A were present not only in plasma (70%) but also in the erythrocytes (30%). From the plasma fraction, about 15% of CAs were bound to plasma proteins. If the CAs present in the erythrocytes are taken into account, then the percentage of binding of CAs to plasma proteins was even less (11%). Previous experiments (Zia *et al.*, 1971; Danon and Sapira, 1972; May *et al.*, 1974; Powis, 1975; Teixeira *et al.*, 1979; Sager *et al.*, 1987; Trovik *et al.*, 1992) reported that the percentage of binding of CAs to plasma proteins varied from 14% to 50%. The results of these experiments were calculated as if all CAs were present only in the plasma. If those results are recalculated according to our findings (only 70% of total CAs are present in plasma), the percentage of binding to plasma proteins will be much lower (10–35%). Thus, those authors have overestimated the percentage of CAs bound to plasma proteins and the percentage of unbound CAs, but have underestimated the percentage of total binding, because binding to erythrocytes was previously not taken into account. At first CAs are present in the blood in a free (unbound) form. Thereafter the CAs or their metabolites

bind to plasma proteins and to haemoglobin and therefore were present for a long period (up to 1 month). Binding has been reported to reduce the biological activity of CAs (Powis, 1975). Drugs or some conditions that displace CAs from their binding sites have been found to increase the biological potency of CAs *in vitro* (Powis, 1975) and the same may apply *in vivo*.

Precipitation with ammonium sulphate revealed that the percentage of binding of CA to albumin was higher than to globulin, indicating that albumin is the main binding system for A and NA in sheep plasma. This is in agreement with previous studies performed in humans (Zia *et al.*, 1971; Danon and Sapira, 1972; Powis, 1975; Trovik *et al.*, 1992) and in dogs (Teixeira *et al.*, 1979).

As only substances with a molecular weight above 5000 are excluded from the Sephadex G-25 gel, small molecules like CAs or oligopeptides of low molecular weight are retained, but are not well separated. Therefore, the elution of radioactivity in fraction 12 did not indicate that all CAs were free, as some might be bound to small peptides (for example, glutathione).

The percentages of binding of ^{3}H-NA to plasma proteins obtained with Sephadex gel filtration were only slightly higher than those obtained with acetone precipitation. Acetone was used for the precipitation of plasma proteins because it produces a fine precipitate, which can be easily dissolved and counted. The percentage of binding of ^{3}H-NA to plasma proteins obtained with acetone precipitation was not affected by increasing concentrations of NA or by the order of adding labelled and unlabelled NA. These findings indicate a high binding capacity of the proteins for CAs. They also demonstrate a strong binding of the ^{3}H-CA (metabolites) to the proteins, which was further underlined by the acid precipitation experiment and has been suggested by Teixeira and colleagues (1979). Thus, CAs strongly attached to albumin are probably covalently bound. As mentioned previously, 41% of CAs was bound (about 30% to the erythrocytes and about 11% to plasma proteins) and the other 59% might be either unbound or partly bound to unknown binding systems (such as small peptides). The fact that the radioactivity was also found within the red blood cells suggests a transport system through the membrane as described for human erythrocytes (Azoui *et al.*, 1996). The radioactivity may be bound to haemoglobin ('catecholaminated' haemoglobin) by the same mechanism described for glucose (glycosylated haemoglobin; Khaw *et al.*, 2001).

In the future, efforts should be made to characterize these adduct products (CAs attached to plasma proteins, haemoglobin or small peptides) and to establish an assay to determine them *in vivo*. If this could be achieved, it would yield a valuable tool for measuring stress experienced for a longer period that could be used not only for animals but also for humans.

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