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THE ASSOCIATION OF STEROIDS WITH BLOOD CELLS *IN VIVO*

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SUMMARY

The distribution of steroids between plasma and blood cells in adrenal venous blood or in arterial blood was studied in several mammalian species. Between 20 and 60 per cent of the steroids measured (cortisol, corticosterone, aldosterone, 18-hydroxydeoxycorticosterone, deoxycorticosterone, progesterone and pregnenolone) were found to be attached to the cell fraction. The distribution varied with the chemical nature of the steroids and was different in different species.

INTRODUCTION

THE PRESENCE of steroids in the cell fraction of blood samples was first described in 1932 by Kemp and Bjergaard [1] who found that the oestrogenic activity in the blood of pregnant women was about equally distributed between plasma and blood cells. This observation was confirmed by Albrieux [2]. A more detailed *in vitro* study of the conditions under which oestradiol is taken up by human erythrocytes was carried out by Bischoff and Katherman [3] who found that the distribution coefficient of oestradiol in cells and in plasma is inversely proportional to the solubility of the steroid in the solution in which the cells were suspended. The uptake of cortisol by human erythrocytes was first studied by Peterson *et al.* [4]. They calculated that 20–25 per cent of the cortisol in human blood is associated with erythrocytes assuming a haematocrit of 40 per cent. At the Laurentian Hormone Conference in 1956, Sandberg *et al.* [5] reported that 2 days after the injection of [16-^{14}C]-oestradiol to a woman the concentrations of radioactivity in the blood cells exceeded by far the concentrations in the plasma whereas no significant amounts of radioactivity were found in the cell fraction after the injection of [^{14}C]-testosterone or cortisol. They suggested that the erythrocytes might play a role in the transport of certain steroids in the body.

In human adrenal venous blood Samuels *et al.* [6] found up to 30 per cent of the total 17-hydroxy-steroids associated with the erythrocytes. They were not, however, able to detect cortisol in erythrocytes of peripheral blood. In contrast, Migeon *et al.* [7], after intravenous infusion of 17-hydroxy-steroids, found 17–36·5 per cent of the steroids in peripheral blood bound to blood cells. *In vitro* experiments by Goldzieher, Baker and Nyman [8] on stallion blood incubated with radioactively labelled oestrogens in concentrations ranging from 0·1 ng to 1 mg/ml showed that the distribution of the radioactivity was independent of the concentration and that equilibrium was achieved in less than one hour. Farese and Plager [9] concluded from their observations that the uptake of cortisol by red blood cells in normal subjects is dependent upon the binding of cortisol by plasma proteins and the plasma cortisol concentration.

Thus, by the start of the last decade the association of steroids with blood cells was a well established, but not a well publicised fact. It was then and is even still now ignored by a number of investigators who extract only plasma when measuring blood steroid contents.

Our interest in the problem arose from practical considerations. At the start of experiments on the control of aldosterone secretion in the dog[10], we too, were not familiar with the observations on steroid binding by blood cells and measured only the plasma steroid concentrations after centrifuging the adrenal venous blood at 1700–2000 *g*. One day, however, instead of discarding the cell fraction we lysed the erythrocytes with one volume of water and extracted them, similar to the plasma, 3 times with double the volume of a mixture of ethylacetate and ether 2:1. To our surprise we found that a large percentage of the corticosterone, cortisol and aldosterone was present in the blood cells (Fig. 1).

In another experiment we compared the quantities of steroids extracted from the plasma and cell fraction of an adrenal blood sample with that extracted from a portion of whole blood from the same adrenal blood sample and found that only part of the aldosterone expected to be present in the cells was extracted by the method used (Fig. 2). However, if the cells were either diluted with an equal volume of peripheral plasma, haemolysed with double the volume of water and extracted with 4 volumes of the organic solvents (Fig. 3) or haemolysed in three volumes of water instead of one (Fig. 4), a good agreement was obtained between the quantities of a steroid found in whole blood and the sum of the amounts of this steroid in the plasma and in the cell fraction.

These observations led to the following experiments on the association of blood cells with steroid hormones *in vivo*. Most of them were carried out on adrenal venous blood which was collected in polyethylene tubes kept in ice water. The animals were under chloralose or sodium pentobarbitone anaesthesia and heparin had been injected. The whole blood and plasma samples were diluted with an equal volume of water and the packed cells with 3 volumes of water before the extraction with the ethylacetate-ether mixture. The samples were purified by solvent partition and the individual steroids separated by paper chromatography before and after derivative formation. Cortisol, corticosterone and aldosterone were finally measured by their reaction with blue tetrazolium[11]. Pregnenolone, progesterone, deoxycorticosterone and 18-hydroxy-deoxycorticoster-

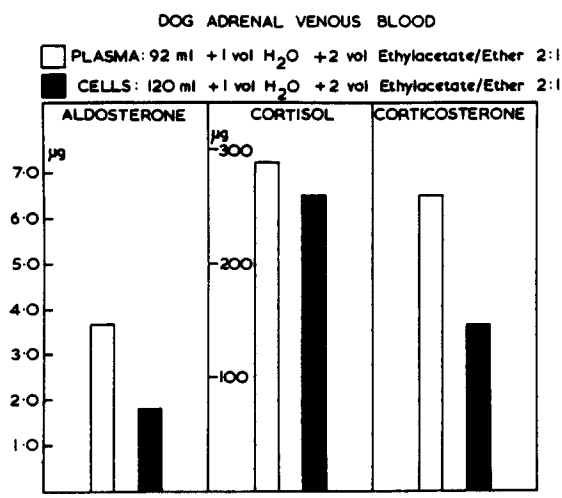

Fig. 1. Distribution of steroids in dog adrenal venous blood. Insufficient extraction of cell fraction.

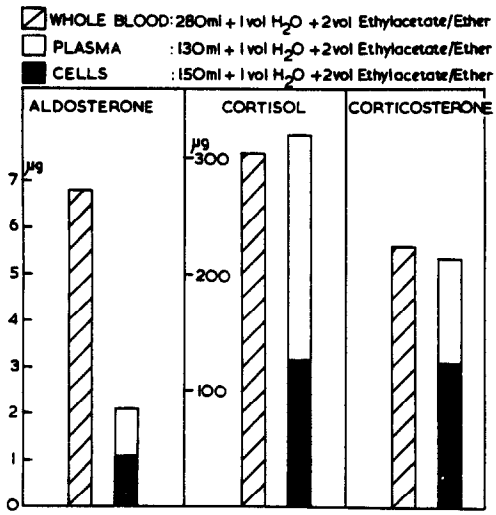

Fig. 2. Comparison of steroid content of a portion of whole dog adrenal venous blood and of the plasma and erythrocyte fraction of the same blood sample. Cells insufficiently extracted.

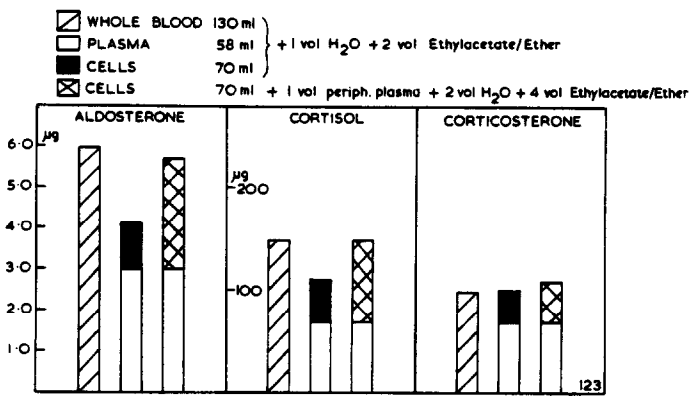

Fig. 3. As Fig. 2. In addition, one part of the erythrocyte fraction extracted after dilution with peripheral plasma.

one were estimated by gas liquid chromatography[12]. Some of the earlier experiments were done in collaboration with Vogt[13]. Dr. Birmingham (McGill University, Montreal) participated in one of the later experiments.

DISTRIBUTION OF STEROIDS BETWEEN PLASMA AND CELLS IN ADRENAL VENOUS BLOOD OF DIFFERENT SPECIES

Observations made on adrenal venous blood of the dog, the guinea-pig, the rat and the rabbit are illustrated in Fig. 5. There is a clear difference in the binding of steroids by the blood cells between the species. Whereas one half of the major steroids secreted by the dog adrenal (cortisol and corticosterone) and the guinea-pig adrenal (cortisol) were present in the blood cells, only 25 per cent of the major steroid (corticosterone) secreted by the rat and rabbit were found in the cells. In *in vitro* experiments Ohtsuka and Koide[14] found that the uptake of radioactive

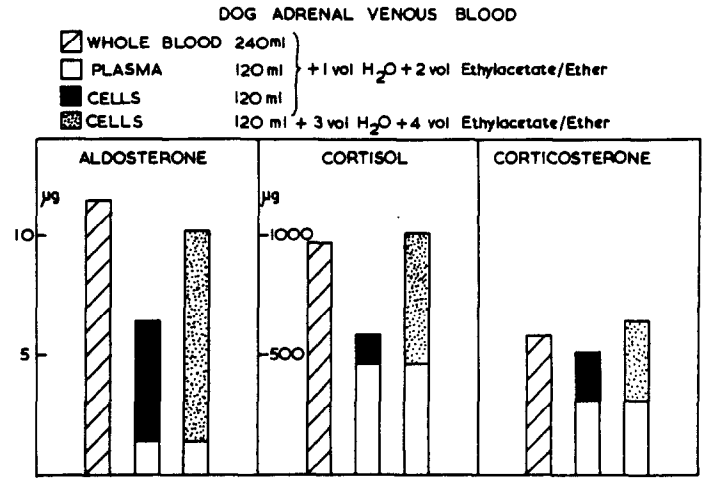

Fig. 4. As Fig. 3. One part of the erythrocyte fraction extracted after dilution with 3 volumes of water.

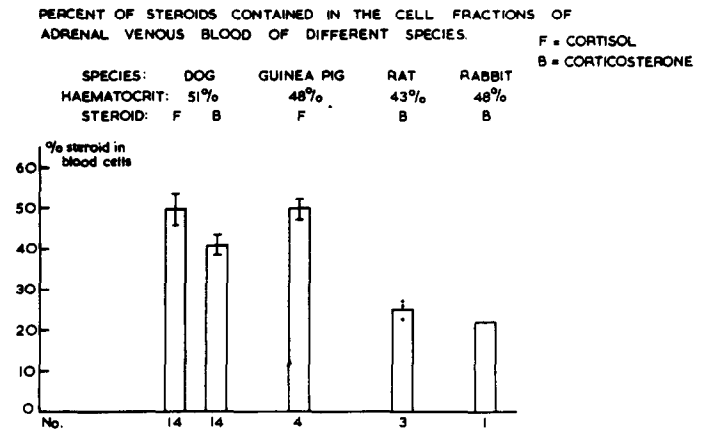

Fig. 5. Species differences in the blood cell binding of the major steroids in adrenal venous blood.

aldosterone, progesterone, testosterone and DHA was higher by human and dog erythrocytes than by those from a Pekin duck. The latter bound more oestrogens and glucocorticoids.

Exceptions which were sometimes seen within a species were in part due to large differences in the haematocrits, which not only changed the total amounts of steroids present in the two fractions but also the concentrations of the steroids in each fraction. The distribution of steroids can also be influenced by other factors, like the composition of the plasma proteins[9, 15]. Differences between the specific steroid binding proteins might contribute to the species differences shown in Fig. 5.

DISTRIBUTION OF DIFFERENT STEROIDS BETWEEN PLASMA AND CELLS OF ADRENAL VENOUS BLOOD

In Fig. 6 the concentrations of cortisol, corticosterone and aldosterone in the adrenal venous blood of 4 dogs are illustrated. In 3 of these 4 animals the cell

concentrations of cortisol and corticosterone were lower than the plasma concentrations whereas aldosterone was fairly equally distributed. In the dog experiment shown in Fig. 4, however, the concentration of aldosterone in the cells was nearly four times that in the plasma, whereas cortisol and corticosterone were equally distributed.

In the adrenal venous blood of a rat (Fig. 7), the plasma concentration of corticosterone was about three times that of the cell concentration. For 18-hydroxydeoxycorticosterone and deoxycorticosterone this factor was only 1·5 and 2, respectively, whereas the concentration of pregnenolone was the same in plasma and cells. In an adrenal blood sample from another rat we found that the concentration of progesterone in the plasma was 0·04 μg/ml, in the cells 0·11 μg/ml

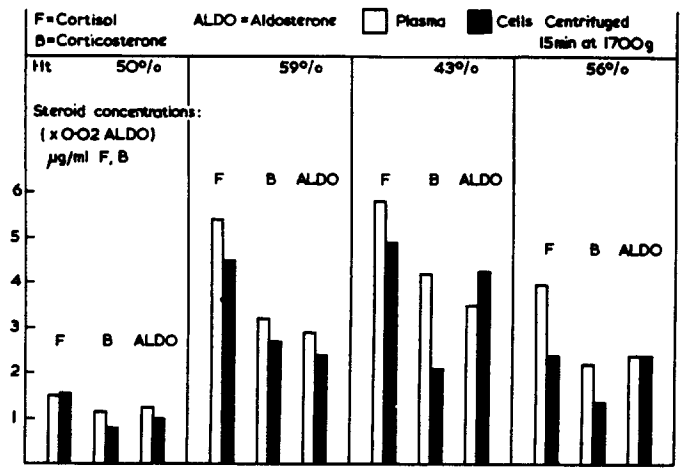

Fig. 6. Distribution of different steroids in the adrenal venous blood of 4 dogs.

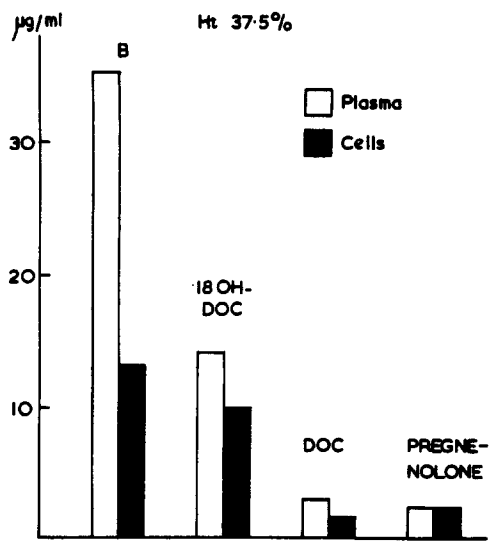

Fig. 7. Distribution of different steroids in the adrenal venous blood of the rat.

(haematocrit: 33 per cent). Figure 8 shows the gaschromatography tracing obtained from the eluate of the pregnenolone region of a paper chromatogram on which the extract of the adrenal venous blood cells of this rat was developed. In *in vitro* studies on rat erythrocytes De Venuto[16] observed that 70–85 per cent of the total progesterone in contact with the cells was taken up. The uptake of aldosterone was insignificant. In the adrenal blood of a guinea-pig the concentrations of cortisol in plasma and in the cells was equal whereas the pregnenolone concentration in the cells was 1·5 times that in the plasma (Fig. 9). Different

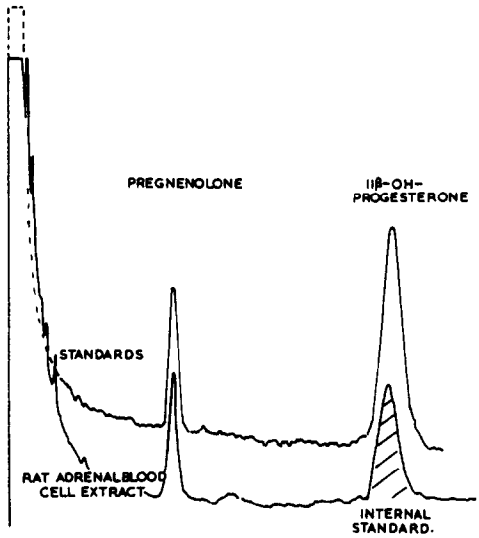

Fig. 8. Gaschromatography tracings. Bottom tracing: eluate of the pregnenolone region of a paper chromatogram on which the extract of adrenal blood cells of a rat was developed (F + M gaschromatograph, 120 cm 3·8% SE-30 column 230°C). Top tracing: standard steroids.

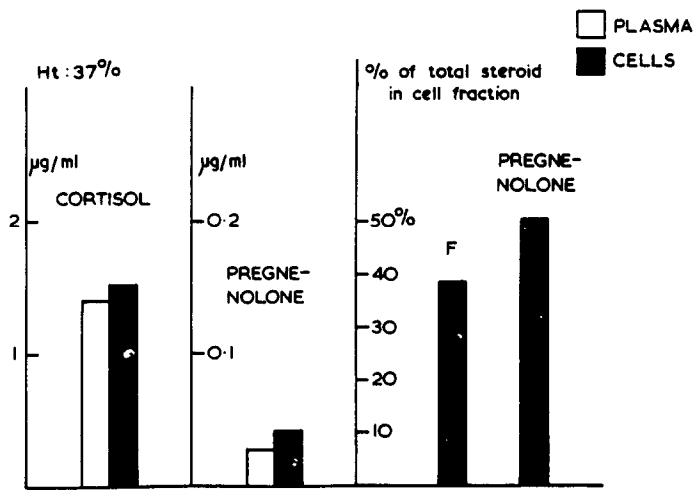

Fig. 9. Distribution of cortisol and pregnenolone in the adrenal venous blood of the guinea pig (Ht = haematocrit).

affinities of human erythrocytes for different steroids was observed by Sandberg *et al.*[5]. They found that the cells were able to take up a large proportion of injected oestradiol but not of testosterone or cortisol. Bartter and Slater[15] made similar observations in *in vitro* experiments on dog erythrocytes. The observations on the blood samples could be explained by different binding affinities and capacities of specific plasma proteins binding different steroids. This would be in accordance with the suggestion of Pinsker *et al.*[17] that it is the ultrafiltrable fraction of the steroid which combines with the erythrocytes. In order to explain the *in vitro* observations, however, one has to postulate that the erythrocyte itself possesses different binding capacities for different steroids.

WHAT IS THE EVIDENCE THAT THE BINDING OF STEROIDS TO BLOOD CELLS TAKES PLACE *IN VIVO*?

Several experiments were carried out in order to investigate whether the association of steroids with blood cells is the same inside the body and after the blood left the organism. Dog adrenal venous blood was centrifuged 1–20 min after the start of the blood collection. One half of the plasma and the cells obtained were extracted separately, the remainder was combined and allowed to stand for 2 h at +3°C. No significant uptake or loss of any of the steroids measured by the cell fraction could be observed during this time (Figs. 10 and 11). In one early experiment[13] a migration of aldosterone from plasma to cells during standing in the cold was seen in a dog adrenal blood sample. In *in vitro* experiments the cell concentration of cortisol and aldosterone was maximum within 1 min and did not change during the following 90 min at room temperature[15]. As can be seen in Fig. 10, no significant loss of any of the steroids measured occurred during standing at +3°C.

As it appeared possible that the entry of steroids into the cell fraction was favoured by cooling the blood as soon as it left the body a sample of dog adrenal blood was collected for 5 min at +37°C and spun immediately. Another sample from the same dog was collected at +2°C. In both samples 50 per cent of the cortisol was found in the cells.

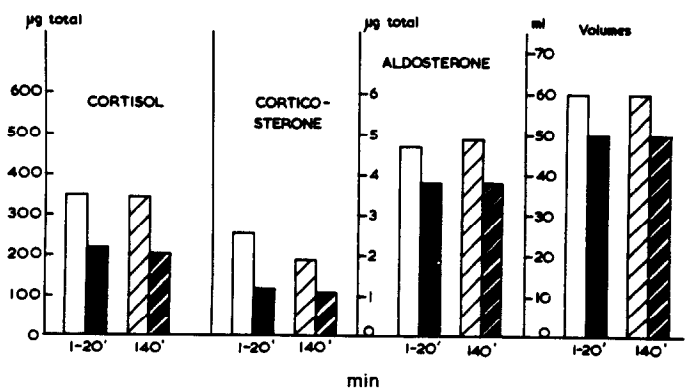

Fig. 10. Distribution of steroids between plasma and cells of dog adrenal venous blood after short and long combination of the two fractions. Plasma and cells were combined for 1–20 min (□, ■) or for 140 min (▨, ▨) at +3°C.

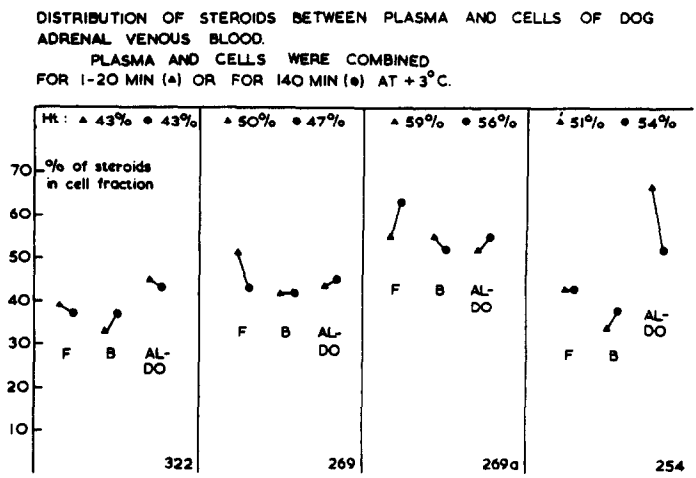

Fig. 11. As Fig. 10. F = cortisol; B = corticosterone; ALDO = aldosterone.

Inhibition of glycolysis by the addition of sodium fluoride during the collection did not affect the distribution of steroids between plasma and cells.

These experiments provide evidence for the assumption that the distribution of steroids between plasma and cells of adrenal venous blood is the same within the body as after collection in the test tube.

STEROIDS IN CELLS OF PERIPHERAL BLOOD

In the arterial blood of a stressed dog we found 4·8 μg of cortisol in 100 ml of packed cells and 3·9 μg of cortisol in 100 ml of plasma. Cortisol infused into the jugular vein of a dog (15 kg) at a rate of 500 μg/min for 40 min increased the cortisol concentrations in the plasma and in the cells of the blood drawn from the aorta of the animal (Fig. 12).

Ten minutes after the intravenous injection of ^{14}C-labelled cortisol into a rat (0·2 μCi/kg body wt.) about 28 per cent of the total radioactivity in the blood from the carotid artery could be extracted from the erythrocytes. This is similar to the percentage in which endogenous corticosterone is found in the cells of adrenal venous blood of the rat (Fig. 13). Vermeulen[18] infused ^{14}C-labelled cortisol into human subjects and found 16–37 per cent of the radioactivity in the erythrocyte fraction.

In two experiments on guinea-pigs either the blood cells collected from the adrenal vein were allowed to equilibrate with an equal volume of plasma from the carotid artery of the same guinea-pig for 10 min at 37°C, or blood cells from the carotid artery were equilibrated in the same manner with an equal volume of adrenal venous plasma. In both experiments the cortisol concentrations in plasma and cells were similar after the incubation (Fig. 14). No metabolism of cortisol was apparent during this time as the total amount of cortisol remained unchanged throughout the experiment. In both guinea-pigs the peripheral steroid concentrations were high, due to the prolonged stress of laparotomy and adrenal blood collection.

The presence of steroids in erythrocytes of peripheral blood and the uptake of infused exogenous steroids by the blood cells, demonstrate clearly a transport function of these cells.

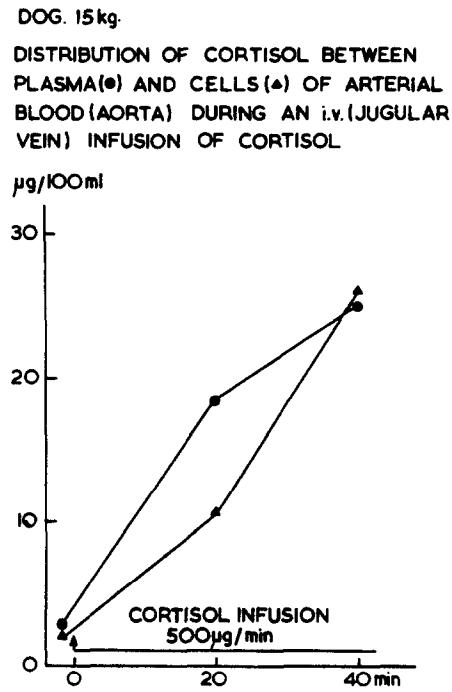

Fig. 12. Uptake of intravenously infused cortisol by the blood cells of arterial blood of the dog.

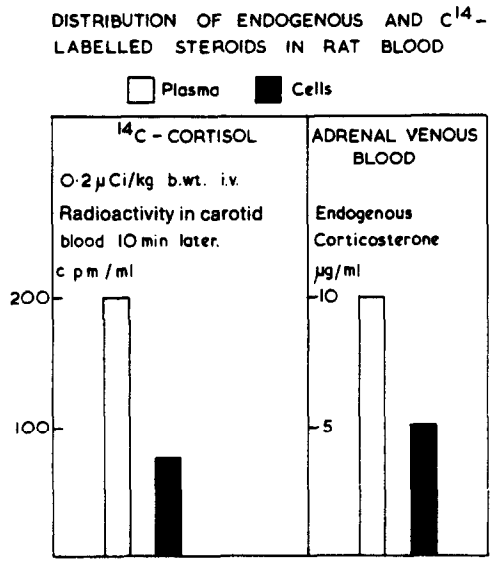

Fig. 13. Uptake of intravenously infused, ^{14}C-labelled cortisol by the blood cells of carotid blood of the rat.

REMOVAL OF CORTISOL AND CORTICOSTERONE FROM BLOOD CELLS BY WASHING WITH SALINE

In two experiments cells from dog adrenal venous blood were suspended in three times their volume of a 0·9 per cent solution of sodium chloride and gently

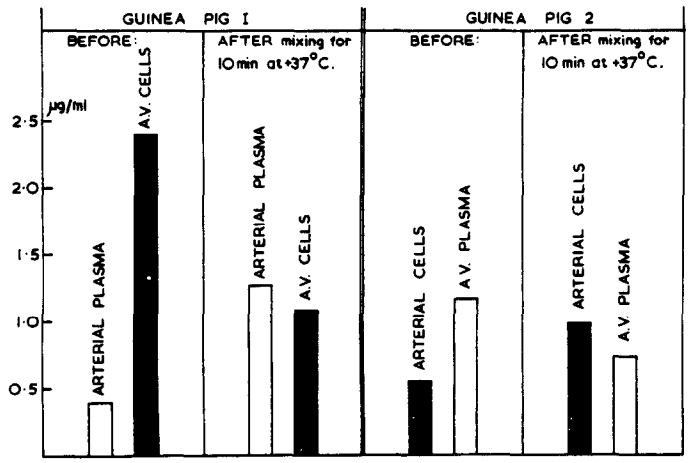

Fig. 14. Uptake of cortisol by peripheral plasma from the blood cells of adrenal venous (A.V.) blood and by peripheral blood cells from adrenal venous plasma. Equal volumes of plasma and cells were mixed.

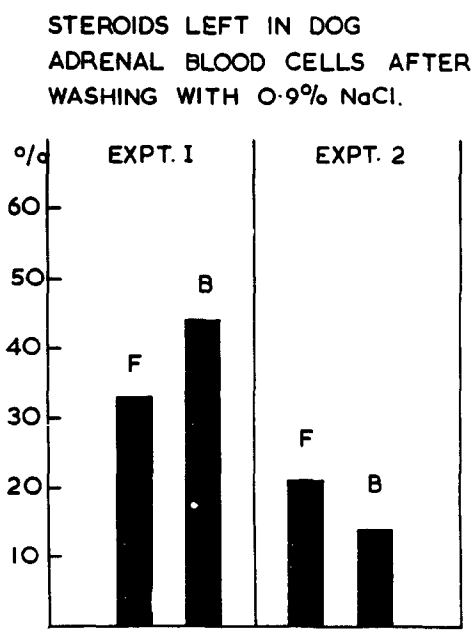

Fig. 15. Effect of washing with 0·9% NaCl on the steroid concentration in erythrocytes from dog adrenal blood. (F = cortisol: B = corticosterone). 100% = steroid content of cells before washing.

shaken for 15 min. After centrifugation at 2000 *g* the cells were washed a second time and again separated. The results (Fig. 15) show that part of the steroids can be detached from the cells by this procedure.

TYPE OF BLOOD CELL TO WHICH STEROIDS ARE BOUND

From *in vitro* experiments it is well established that washed erythrocytes, and even fractions of erythrocytes[19] are able to bind steroids. Therefore there is

little doubt that the majority of the steroids found *in vivo* in the cell fraction of centrifuged blood samples will be attached to erythrocytes. The possibility exists, however, that other cellular elements may also accumulate steroids. As 5-hydroxytryptamine and catecholamines are easily taken up by thrombocytes and can be accumulated in these cells against a concentration gradient, we also investigated whether steroids are contained in thrombocytes. No corticosterone ($< 0{\cdot}05\ \mu g$) was found in the extract of thrombocytes which were isolated from 7·5 ml of adrenal venous blood of a rat. It is feasible that the situation is different for other steroids, e.g. oestrogens which have a phenolic hydroxyl group. It will be of interest to study whether white blood cells can bind steroids, especially since the number of these cells in the circulation is affected by steroid hormones.

One practical consequence of the steroid binding by erythrocytes is the necessity to measure steroid concentrations in whole blood samples instead of plasma if parameters like steroid secretion rates from endocrine glands or metabolic clearance rates are studied. Many investigators hesitate to use whole blood samples as slightly more work is involved in the purification. In some cases it might be possible to obtain a value for the content of a steroid in whole blood if small quantities of this steroid in a radioactively labelled form are mixed into the blood before centrifugation. This is based on the assumption that the affinity of the erythrocytes in the test tube for a steroid in ethanolic solution is the same as for the endogenous steroid. For progesterone this assumption seems to hold (Fig. 16).

In order to fully understand the physico–chemical processes involved in the steroid binding by erythrocytes much work has still to be done. The steroid hormones enter the circulation in the plasma and attached to blood cells. The binding to the cells occurs in all probability already within the adrenal gland. If the steroids extracted from the blood cells were merely contained in the very small plasma fraction trapped between the cells, then the steroid concentration in this plasma fraction would be up to 100 times higher than the concentration in the plasma separated off by centrifugation. In this case it would be necessary to

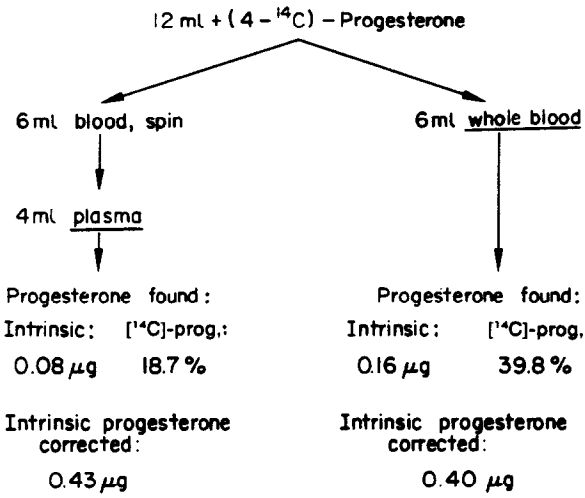

Fig. 16. Rat adrenal venous blood. Estimation of progesterone in plasma and in whole blood after addition of [4-^{14}C]-progesterone to the blood.

postulate the existence of a special plasma protein with a very high steroid binding capacity which remains trapped between the erythrocytes.

In the circulation the steroids are present in several physical forms: they are bound to specific proteins as e.g. transcortin, to other plasma proteins like albumin, and to red blood cells, or they are free in solution. An equilibrium must exist between these forms. As Farese and Plager[9] pointed out, the amount of steroid present in each form is dependent on the relative binding affinities, the binding capacities and the total amount of steroid present. Studies of all these factors in the same blood samples will shed some more light on the role which the erythrocytes play in the transport of steroid hormones. The binding sites on the erythrocytes can be studied *in vitro*. Such experiments have been carried out by Brinkmann *et al.*[19] with human erythrocytes. Their studies with pregnenolone, progesterone, 20α-hydroxy progesterone and testosterone indicated that a large percentage of the steroids was bound to proteins of the membrane free haemolysate.

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DISCUSSION

Kellie: I wonder if I could ask three questions. First, after the red coils have been spun down, are they resuspended in isotopic saline so that they are washed before they are processed? Second, do any of the experiments that you have carried out reveal whether the steroids are adsorbed externally on the erythrocytes, or alternatively whether they are in fact inside? Third, is there any evidence that the steroids that you used are modified by this process of transfer to the erythrocytes?

Holzbauer: In answer to your first question: The cells we have studied were not washed with saline. We have carried out several experiments in which packed erythrocytes were resuspended in three times their volume of 0·9% sodium chloride and gently shaken for 15 min at +21°C. The samples were then centrifuged (2000 *g*, 20 min) and the cells washed once more in saline. The results of 2 experi-

ments on erythrocytes from dog adrenal venous blood are shown in Fig. 15. We found that up to 45% of the steroids originally found in the cell fraction can remain attached to the cells after this washing procedure. It may be possible that the erythrocytes could lose all their steroids against a concentration gradient if they were suspended much longer in a larger volume of saline. These are, however, artificial conditions which do not give any information on the amount of steroids which are attached to the blood cell within the blood stream in the body.

In answer to your second question: The experiments of Dr. van der Molen and his colleagues have shown that haemoglobin is able to bind steroids. Dr. K. Matthews in Cambridge has compared the uptake of cortisol by ghosts of human erythrocytes and by intact erythrocytes. Per unit weight he calculated that the empty erythrocyte membrane has taken up only 2% of the amount of steroids which were taken up by the intact cells.

To your third question: In slide 10 (Fig. 10). The results of an experiment were shown in which the steroid content of cells from dog adrenal venous blood was shown 20 min after the blood collection and also 2 h after standing at +4°C. There was no indication of any loss of cortisol, corticosterone or aldosterone during these 2 h at +4°C. Bartter, F. C. and Slater, J. D. H. (*J. Physiol. (London)* **184** (1966) 29P) found that aldosterone which was taken up by dog erythrocytes was not metabolized during 90 min at room temperature. Conversion of oestrone to oestradiol in erythrocytes has been described and also the presence of a C21-dehydrogenase in erythrocytes.

Martini: Did I understand you correctly to say that testosterone is not bound to the red cells?

Holzbauer: We have not studied testosterone uptake and by red cells.

Martini: Because we have opposite results: We have injected labelled testosterone *in vivo*, and found that quite a lot is bound to red cells. Those are Dr. Kniewald's experiments in my lab? May I point out one thing: several years ago, here in Paris, Professor Courrier showed that erythrocytes also bind thyroxine, a result which was presented at one of the Ciba Foundation Symposia in the early 1950's. I remember that nobody accepted those results at that time.

I wanted to ask you another question: can one steroid displace another steroid from the erythrocites? I mean, if you give progesterone, can you displace cortisol, or the reverse?

Holzbauer: Adrenaline and noradrenaline (Roston S., *Nature (London)* **215** (1967) 432) and dopamine (Bryson G. and Bischoff F. *Clin. Chem.* **16** (1970) 312) were also found to be taken up by erythrocytes. We did not do any replacement experiments.

Brinkmann: The results which you found in your time and temperature studies are in good agreement with our *in vitro* incubations of steroids with human erythrocytes suspended in Krebs–Ringer buffer. I would like to ask one question: did you find any correlation between the total steroid concentration in the blood and the uptake of the steroid by red blood cells?

When plasma proteins become saturated with steroid, there may be a redistribution of steroids in favour of the erythrocytes. With erythrocytes suspended in a 5% plasma solution we found an increase in the uptake of progesterone by the red blood cells after saturation of progesterone binding plasma proteins.

Holzbauer: From our observations it appears, that *in vivo*, the uptake by erythrocytes of relatively large amounts of one steroid, e.g. cortisol, which is present in

the blood in high concentrations, does not interfere with the uptake of another steroid, e.g. aldosterone, which is present in much lower concentrations.

Carstensen: I have done some experiments on the distribution of testosterone between cells and plasma in human and rat blood. We find about 10–30% testosterone in the red blood cell fraction. We have measured the distribution in haematocrit tubes after centrifugation at 11,000 *g* in order to get Koeppe's criterion, so I believe that there is very little trapped plasma. Another point which I would like to stress is that the erythrocytes may change their properties if they are washed. This is indicated by the effect of repeated washing: it becomes very difficult to remove the last traces of testosterone. We did the following experiment: we took ½ ml of pure red cells and washed them repeatedly with 1 ml of saline, and found that between 10 and 12 washings were needed to remove the testosterone completely. When we did this with tritiated testosterone, we found that the bound to unbound ratio increased. This was down to about 5 pg of testosterone. The same experiment done by adding increasing amounts of testosterone, over the same range of concentrations, to fresh cells results in an unchanged ratio of bound to unbound steroid. So the membrane probably changes its properties during washing.

Slater: Dr. Kellie, with regard to your question about whether steroids penetrate the red cells or whether they just sit on the surface, the volume of distribution of cortisol, corticosterone and aldosterone when cells are suspended in saline is virtually indistinguishable from the volume of red cell water. This is not complete evidence, but DHA will inhibit the glucose-6-phosphatase contained in intact cells so that presumably DHA penetrates the cell membrane.

Kellie: Will DHA sulfate do this?

Slater: I don't know about that. May I ask whether Dr. Holzbauer feels that many of her results, in particular the differences in the amounts of the various C_{21} steroids which go into red blood cells, can be explained as a function of the concentration of available high-energy binding sites in plasma proteins? Certainly our experiments were explicable in terms of the likely concentration of high-energy binding sites in plasma (*J. Physiol.* **184** (1966) 29–31P).

Holzbauer: The distribution of steroids between plasma and cells must depend on the steroid binding capacity and affinity of the proteins contained in the plasma in which the cells are suspended and on the binding capacity and affinity of the cells themselves. These parameters are seemingly different in different species and depend also on the chemical structure of the steroid studied.