In vitro Studies on Distribution of Cortisol in Dog's Blood.* (31131)

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Studies on the distribution of cortisol (4-pregnen-11β, 17α, 21-triol-3, 20-dione) between plasma and blood cells have led to many conflicting reports concerning the amount of steroid taken up by these cells and the time required for this association to occur. The percentage of blood cortisol found in and/or on erythrocytes has been reported to vary from 10-37%(1-4). Wu and Mason(3) observed even larger quantities in blood cells which they attributed to nonspecific chromogens in the analyses of 17-OHCS (17-hydroxycorticosteroids). To prevent the penetration of plasma 17-OHCS centrifugation of blood immediately upon sampling has been proposed(4); however, Bush(5) has demonstrated that although cells begin to take up cortisol immediately upon its addition to blood, an equilibrium is not established for at least 2 hours. On the other hand, others have shown that, once blood is withdrawn from the subject, neither time nor temperature is a factor in the distribution of blood cortisol(3,6). This equilibrium may not only be due to the uptake of steroids by cells, but also to the ability of steroids to reenter the plasma when the latter levels are low. Thus, it has been shown that cortisol(5) and 17α-estradiol(7) can pass freely from erythrocytes into the suspending media.

Since there has been considerable disagreement concerning the role of blood cells in the transport of adrenocortical steroids, a series of *in vitro* experiments was undertaken to ascertain 1) time required for cortisol added to whole blood to equilibrate between blood cells (mainly erythrocytes) and plasma, 2) ability of 17-OHCS to reenter the plasma from blood cells which were obtained from animals infused with large quantities of cortisol, and 3) relationship of increasing red cell volumes to the disappearance of 17-OHCS from plasma.

Materials and methods. The following 3 experiments were performed: *Exp. A*: Incubation time. Heparinized blood was rapidly collected from the femoral arteries of 4 mongrel dogs anesthetized with sodium pentobarbital. After obtaining blood for microhematocrits one-half of the blood was rapidly centrifuged at room temperature. Two 100 ml aliquots of both whole blood and plasma, taken from the same animal, were placed in Erlenmeyer flasks and gently shaken in a 37°C water bath. Cortisol (250 μg) dissolved in 0.4 ml 5% ethanol-saline was added to one flask of whole blood and to one of plasma, while a similar volume of 5% ethanol-saline was added to the remaining 2 flasks. At 0, 2, 30, 60, 90 and 120 minutes 10 ml aliquots were removed from each flask and immediately centrifuged before storing the plasma at —25°C. *Exp. B*: Release of 17-OHCS by blood cell. After cannulating the right femoral artery and cephalic vein in each of 4 anesthetized dogs, heparin (250 units/kg) was given intravenously and approximately 120 ml blood (normal erythrocytes = NE, and normal plasma = NP) were removed from the artery. Cortisol (1 mg/kg body weight) was then rapidly infused into the cephalic vein and after 5 minutes 200-250 ml of arterial blood (infused erythrocytes = IE, and infused plasma = IP) were collected. Micro-hematocrits were performed on both normal and infused blood in order to reestablish the original red cell and plasma volumes when making the following 6 combinations: 1) NP, 2) NP with NE, 3) NP with IE, 4) IP, 5) IP with IE, and 6) IP with NE. Two and 30 minutes after combining the various phases and gently shaking in a 37°C water bath, the samples were rapidly centri-

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fuged and the plasma stored in a freezer prior to analysis. *Exp. C*: Hematocrit variations. Four hundred ml of blood were rapidly removed from the femoral artery of each of 6 anesthetized dogs. Micro-hematocrits were determined and blood samples containing red cell volumes ranging from 20 to 50% in increments of 5% were obtained by either adding or removing calculated amounts of plasma collected from the same animal. Cortisol was added at each hematocrit (0, 20, 25, 30, 35, 40, 45 and 50%) and at 2 concentrations (25 or 250 μg/100 ml). After incubating in a 37°C water bath for 30 minutes, micro-hematocrits were performed to verify the calculated red cell and plasma volumes. Final hematocrits did not vary more than 1.5% of calculated amounts.

The concentrations of free 17-OHCS were determined in plasma samples obtained from all 3 experiments by a modification of the Porter-Silber reaction(8) and the quantities of 17-OHCS in blood cells were calculated. A few blood samples in which hemolysis occurred were discarded.

Results. The concentrations of 17-OHCS observed in both plasma incubated with ethanol-saline and whole blood similarly treated ranged from 20.5 ± 2.0 to 24.3 ± 1.8 and 19.7 ± 2.1 to 22.2 ± 1.8 μg/100 ml plasma, respectively (Fig. 1). The lack of significant differences (P>.20) within as well as between the ranges indicated that incubating for 2 hours and the presence or absence of blood cells had no discernible effects on the endogenous concentrations of 17-OHCS/100 ml plasma. These basal values for plasma 17-OHCS are admittedly high; however, it must be emphasized that to obtain the large volumes of blood required for these experiments, the animals were rapidly hemorrhaged and the latter is known to markedly increase peripheral plasma 17-OHCS levels (9).

After correcting for 17-OHCS concentrations found in plasma incubated with ethanol-saline, the recovery of cortisol (250 μg) added to 100 ml plasma ranged from 98-102% over the 2 hour period (Fig. 1). Likewise, when the concentrations of 17-OHCS/100 ml plasma observed in 100 ml whole

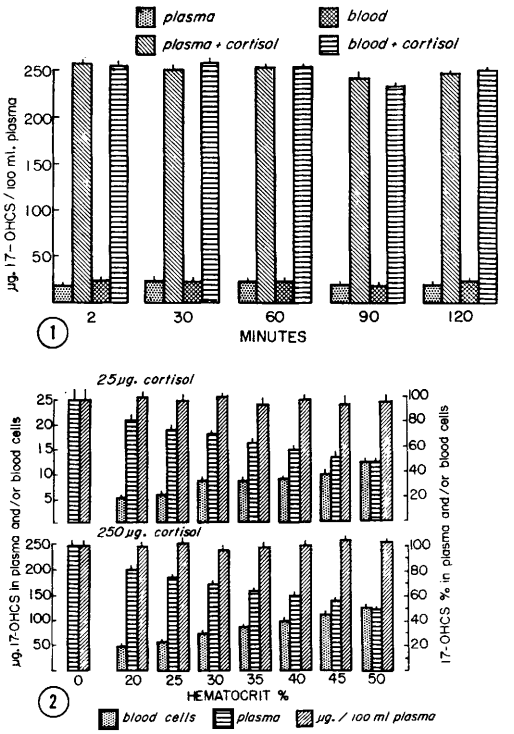

FIG. 1. Effects of addition of 250 μg cortisol dissolved in 5% ethanol-saline to 100 ml of blood or plasma on μg 17-OHCS/100 ml plasma (Mean ± S.E.) during a 2-hr incubation period at 37°C. Control plasma and blood samples were incubated with ethanol-saline.

FIG. 2. Effects of addition of 25 and 250 μg cortisol to 100 ml of plasma and whole blood with hematocrits ranging from 20 to 50% on the observed μg 17-OHCS/100 ml plasma. Percentages and μg 17-OHCS in plasma and red blood cells of 100 ml blood were calculated from their respective volumes.

blood incubated with 250 μg cortisol were corrected for blood with ethanol-saline levels, the values ranged from 243 ± 6 to 256 ± 3 μg/100 ml plasma. These data indicate that time did not affect the uptake of 17-OHCS by blood cells and further, when these levels are reported as μg/100 ml plasma, they are not significantly different from those observed in plasma incubated with cortisol. If the 250 μg cortisol which were added to whole blood (36 ± 2 ml red cells; 64 ± 4 ml plasma) had remained solely within the plasma, then approximately 390 μg/100 ml plasma (250/64 × 100) or 250 μg/64 ml plasma should have been contained within the plasma instead of the observed 251 μg/100 ml plasma (mean over 2 hour incuba-

TABLE I. Distribution of Free 17-OHCS Between Various Combinations of Plasma (P) and Erythrocytes (E) when Using Blood* Taken from Animals With (I) and Without (N) Cortisol Infusions.

Contents	17-OHCS, μg/100 ml plasma 2 min	30 min
NP	16 ± 3†	16 ± 3
NP + NE	15 ± 3	16 ± 3
NP + IE	75 ± 3	74 ± 4
IP	164 ± 8	163 ± 9
IP + IE	167 ± 5	168 ± 7
IP + NE	98 ±10	101 ±11

* 37 ± 2 ml red blood cells/100 ml blood.
† Mean ± S.E.

tion). Based on red cell and plasma volumes in 100 ml whole blood, the calculated amount of 17-OHCS associated with red cells during the 2 hour period was 36% (88 ± 4 to 92 ± 4 μg) while 64% (155 ± 4 to 164 ± 5 μg) remained in plasma. Thus, the data suggest that when cortisol is added to equal volumes of whole blood or plasma, it is rapidly diluted in a similar fashion regardless of the presence or absence of blood cells. The ethyl alcohol within which the cortisol was dissolved did not affect the distribution of 17-OHCS, since a few experiments using cortisol in saline alone gave similar results.

The data on the distribution of 17-OHCS between various combinations of plasma and erythrocytes (Table I) revealed no significant differences ($P>.20$) between levels observed at 2 and 30 minutes for all combinations nor between NP with and without NE, and between IP with and without IE. The difference between NP (16 μg) and NP with IE (75 μg) indicates that plasma concentration increased 59 μg/100 ml plasma by addition of red cells obtained from cortisol infused animals. This increase is far too large to be accounted for by the small amounts of plasma trapped among the infused red cells. On the other hand, the difference between IP (164 μg) and IP with NE (98 μg) suggests that 66 μg/100 ml plasma were taken up by the erythrocytes. Based on the results of the incubation experiments which indicate that 17-OHCS are freely distributed in whole blood, it is evident that the amount of 17-OHCS associated with erythrocytes is similar to the % hematocrit × plasma concentration/100 ml. Since the mean red cell and plasma volumes/100 ml whole blood were 37 and 63 ml, respectively, calculations of the amount of 17-OHCS/100 ml expected in plasma would be as follows:

For NP with IE: IP (164) × 37% = 61
NP (16) × 63% = 10
71 μg

For IP with NE: NP (16) × 37% = 6
IP (164) × 63% = 103
109 μg.

Neither of these calculated values is significantly different from those (75 and 98 μg) observed, suggesting that 17-OHCS are capable of freely passing into as well as out of blood cells very rapidly.

That the percentages of cortisol which disappear from plasma are directly proportional to red cell volumes is illustrated in Fig. 2. If red cells have a greater affinity for 17-OHCS than plasma, then the μg/100 ml plasma would decrease as red cell volume increases and, conversely, the μg/100 ml plasma would increase as cell volume decreases if plasma has a greater affinity for cortisol than red blood cells. However, since analyses of plasma showed that the μg/100 ml were similar for both plasma alone and whole blood (hematocrits = 20 to 50%) incubated with 25 or as much as 250 μg cortisol/100 ml, the data indicate that 17-OHCS are equally distributed throughout the entire volume. Therefore, in whole blood with 25 μg cortisol, the amounts of plasma 17-OHCS decreased from 21 to 12 μg as plasma volumes decreased from 80 to 50 ml, while the 17-OHCS associated with red cells increased from 5 to 12 μg as hematocrits increased from 20 to 50%. A similar pattern was observed when 250 μg cortisol were added to whole blood with varying hematocrits (Fig. 2).

Discussion. These *in vitro* experiments demonstrate that cortisol (25 to 250 μg/100 ml) incubated with dog's blood is rapidly (within 2 minutes) taken up by blood cells in an amount essentially equivalent to the per cent hematocrit. However, in discussing these results it must be emphasized that the method employed(8) measures free steroids,

namely unconjugated as well as protein bound 17-OHCS. Recently Knigge and Hoar(10) reported that the *in vitro* incubation of cortisol (up to 24 hours) did not result in the formation of protein bound 17-OHCS, but its intraperitoneal administration resulted in marked elevations in protein bound steroids indicating an *in vivo* mechanism. Since the experiments performed with infused blood, which undoubtedly contained some protein bound 17-OHCS, revealed an association between plasma and blood cells similar to that observed in experiments where free cortisol was added *in vitro*, the data suggest that protein bound and unconjugated 17-OHCS are similarly distributed. Based on these observations, it is obvious that the levels of free 17-OHCS appearing in the literature as μg/100 ml plasma can be reported as μg/100 ml blood.

The probable causes for others observing lower percentages of 17-OHCS in blood cells may be one or more of the following: 1) extraction of substances from erythrocytes which may inhibit the development of the spectrophotometric reaction used for steroid analyses, 2) inefficient extraction of some 17-OHCS which may be tightly bound to the cell membranes, 3) washing the cells prior to organic solvent extraction which is known to remove some of the steroids from the cells (5,6), and 4) utilization of steroids by the cells. Many of these difficulties were avoided in the present investigation since washing was not performed prior to analyses of plasma 17-OHCS in whole blood and these values were compared to those observed in plasma similarly treated. Many of the studies appearing in the literature(1-4) were performed on whole blood to which no cortisol or very minute quantities of cortisol were added in order to remain within physiological ranges. At these low levels it is obvious that a slight loss of 17-OHCS during steroid analysis of blood cells would lead to a large percentage error. In addition, the lack of significant differences among 17-OHCS values observed in plasma incubated with or without blood cells suggests that cellular utilization over 2 hours is negligible.

These experiments have not shed any light on the association of cortisol with blood cells. The question still remains as to whether the steroid is in and/or on the cell. The rapidity with which 17-OHCS can attain equilibrium in various combinations of plasma and erythrocytes containing varying amounts of 17-OHCS suggests an attachment to the surface. Other supporting evidence for attachment is that since erythrocytes contain approximately 55-60% effective water volume at least some of the steroids are attached to the surface else one must assume that blood cells are capable of concentrating 17-OHCS. However, the observation that fluoride inhibits the association between plasma and erythrocytes(5) indicates that this phenomenon may be due to both active entrance into the cell as well as adhering to the cell membrane.

Summary. A series of *in vitro* experiments on the distribution of free cortisol between plasma and blood cells reveals that 1) cortisol is rapidly taken up by blood cells at 37°C, 2) the amount associated with red blood cells is essentially equivalent to the per cent hematocrit, and 3) 17-OHCS can leave the cell and enter the plasma when the latter phase contains lower concentrations of these steroids. The results of these experiments suggest that the amount of free steroids associated with cells can be calculated from the analyses of plasma 17-OHCS and that the μg/100 ml blood are essentially similar to the μg/100 ml plasma.

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Blastoid Transformation of Rabbit and Guinea Pig Peripheral Lymphocytes by Phytohemagglutinin.* (31132)

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It is now well established that small lymphocytes from human peripheral blood have a potentiality for growth and differentiation since they can be transformed into cytologically immature "blastoid" cells, capable of mitotic division, when grown in tissue culture with phytohemagglutinin (PHA)(1,2).

Despite the observations with human cells the possibility that a potentiality for blastoid transformation may be a biological characteristic of the mammalian small lymphocyte has heretofore not been studied extensively. Indeed the paucity of data in the published reports(3,4,5) and the conflict of opinions fail to provide convincing evidence that PHA can induce blastoid transformation in small lymphocytes from mammalian species other than man.

In view of the still largely unresolved questions concerning the potentialities of mammalian lymphocytes we have investigated the cytological and biochemical alterations elicited in rabbit and guinea pig peripheral lymphocytes when grown in tissue culture with PHA.

Materials and methods. 1. *Tissue culture.* Blood (30-50 cc) was obtained from rabbits and guinea pigs by cardiac puncture and collected inho heparinized syringes. The blood, from each species, was handled identically except for the method of erythrocyte sedimentation. The rabbit erythrocytes were sedimented with high molecular weight dextran (m.w. 250,000) added in half volume quantities to the heparinized blood whereas the guinea pig erythrocytes were more effectively sedimented by the addition of equal volumes of 3% gelatin. With both methods excellent erythrocyte sedimentation was obtained within 20-30 minutes at 37°C.

The supernatant leukocyte-rich plasma was then aspirated and the cells sedimented at 800 rev/min for 10 minutes. The cell button was subsequently washed 3 times with Eagle's minimal essential medium (Grand Island Biological, Grand Island, N. Y.), resuspended in complete medium (Eagle's MEM, supplemented with 20% fetal calf or rabbit serum and 1% L-glutamine 200 mM) and the total lymphocyte count determined. Replicate cultures each containing 3×10^6 lymphocytes in 4 ml complete medium were incubated in tightly sealed 16 × 150 mm culture tubes at 37°C. Replicate cultures were grown either with Phytohemagglutin (0.1 ml) Type M, Difco Labs, Detroit, Mich., or in its absence, and 2 or more culture tubes harvested at frequent intervals up to 72 hours. The cells were fixed in a mixture of absolute methanol and glacial acetic acid (3:1) and then stained with 1.0% acetic-orecein or Jenner-Giemsa. Most of the slides were examined by phase contrast microscopy which when combined with acetic-orecein staining provided excellent nuclear morphology. One thousand cells on each slide were examined and the percent of typical mature lymphocytes and cytologically transformed cells determined. The percent of transformed cells at each interval of culture was determined by averaging the differential counts from 2 or more replicate cultures. Total cell counts at each period of culture were not determined.

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