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Research Article

IN VITRO STUDIES WITH 16-C^{14}-ESTRONE: DISTRIBUTION BETWEEN PLASMA AND RED BLOOD CELLS OF MAN *

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Albrieux has reported that the blood cells of nonpregnant women contained at least twice as much estrogenic activity as the blood serum, while during pregnancy the estrogenic activity was equally distributed between cells and serum (1, 2); however, the specificity of the assay used in these studies is questionable.

Bischoff, Katherman and Yee, using the uterine weight of immature rats as bioassay technique, investigated the *in vitro* distribution of estrogens in blood (3, 4); after an equilibrium period of 10 minutes at 37.5° C., it was found that the ratio of biological activity in red cells to that in serum of rabbit blood was from 0.39 to 0.11 for estrone. An increase in the total recovery of biologic activity could be produced by prolonging incubation, thus suggesting a partial transformation of estrone into a more biologically active steroid (4); the conditions of this activation were studied in detail (5) and Gray and Bischoff demonstrated quite conclusively that the increase in activity was due to the conversion of estrone to estradiol by human and rabbit red cells (6).

Sandberg, Slaunwhite and Antoniades have observed that approximately 30 per cent of the 16-C^{14}-estrone added to a sample of whole blood remained with the red cells, while in a reverse dialysis experiment 55 per cent of the radioestrone was associated with the red cells (7).

The present work is an attempt to study further the *in vitro* distribution of 16-C^{14}-estrone between plasma and red blood cells of man.

METHODS

Experimental subjects. The heparinized blood samples used in the present study were drawn from normal adult individuals. Subjects B, C and E were females, 24, 31 and 28 years of age, respectively, while Subjects A, D and F were males, 28, 33 and 32 years of age, respectively.

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Centrifugation of the samples. An International Centrifuge, size 2, model V, with a No. 240 head and 100 ml. cups giving a rotating radius of 25 cm. at the tip of the cups was used. All samples were centrifuged at 2,000 rpm for 30 minutes; the relative centrifugal force was approximately 1,100 times gravity at the tip.

Microhematocrits. Duplicate hematocrits were run on each blood sample using an International Micro-capillary Centrifuge (Model 490) and heparinized capillary tubes of 75 mm. in length and 1.0 mm. in diameter. The tubes were centrifuged at 11,000 rpm for five minutes and the percentage of blood cell volume was read on a micro-capillary reader (8).

Purity and standardization of 16-C^{14}-estrone. The 16-C^{14}-estrone was purchased from Charles E. Frosst Co., Montreal, Canada. Its specific activity was 0.729 mc. per mM. The purity of this material was tested as previously described (9); 92 to 95 per cent of the radioactivity of an aliquot chromatographed on paper was recovered in the area of the chromatogram corresponding to an estrone standard.

An ethanolic solution of the steroid was prepared in such a manner that it contained 50,300 cpm per ml.

Mode of extraction of the various specimens. Plasma samples as well as all the other aqueous samples (saline solution, Krebs-Ringer solution) were extracted five times with twice their volume of redistilled chloroform. The chloroform extracts were evaporated to dryness.

The red blood cells were kept overnight in the deep-freeze and then thawed. Three times their volume of 100 per cent ethanol was added and mixed rapidly. The mixture was centrifuged and the supernatants were decanted. The residue was washed with ethanol (equal in amount to the initial red cell volume) and after centrifugation the supernatants were added to those previously prepared and the resultant specimens were subsequently evaporated to dryness.

Paper chromatography and radioautography. Some of the extracts were applied on Whatman No. 2 paper and chromatographed with the system of solvents isooctane: toluene: methanol: water (25:75:80:20) described by De Courcy (10). The R_f values of various estrogens in this system have been reported elsewhere (9). The chromatograms were then radioautographed as previously described (9).

Radioactivity determination. The dry residue of the various extracts was dissolved in a known volume of ethanol and an aliquot was assayed for radioactivity as previously described (11). The assays were made in a

TABLE I

In vitro distribution of 16-C^{14}-estrone between the plasma and the red cells of various blood samples

Subjects*	Sample*	Blood vol.	Blood hemat.	Expected plasma vol.† + 1.3 ml.‡	Obtained plasma vol.	Per cent activity: In obtained plasma vol.	Per cent activity: Expected in plasma§
		ml.		*ml.*	*ml.*		
A	1	30	38	18.6 + 1.3 = 19.9	18.25	74.8	81.5
	2	30	38	18.6 + 1.3 = 19.9	18.0	76.5	84.5
B	1	30	37.5	18.75 + 1.3 = 20.05	18.25	71.7	78.8
	2	30	37.5	18.75 + 1.3 = 20.05	18.25	68.2	75.0
C	1	20	40	12.0 + 1.3 = 13.3	12.5	77.2	82.2
	2	17	40	10.2 + 1.3 = 11.5	11.0	80.0	83.6
D	1	20	44	11.2 + 1.3 = 12.5	11.7	80.0	85.5
	2	20	44	11.2 + 1.3 = 12.5	11.6	82.0	88.4
	3	20	44	11.2 + 1.3 = 12.5	11.9	78.2	82.2

* In Subjects A and B, the blood samples were allowed to stand 30 minutes at room temperature prior to centrifugation, while in Subjects C and D the samples were centrifuged immediately (Experiment No. 1).

† $\left(\text{Blood volume} \times \frac{100 - \text{hematocrit}}{100}\right)$.

‡ Volume of radioestrone solution plus volume of saline solution.

§ Per cent activity expected in plasma equals per cent activity in obtained plasma vol. $\times \frac{(\text{exp. plasma vol.} + 1.3)}{(\text{obtained plasma vol.})}$.

"micromil" window gas flow counter (model D-47, Nuclear, Chicago).

EXPERIMENTS AND RESULTS

Experiment No. 1

A series of 50 ml. centrifuge tubes were prepared containing 0.3 ml. of the standard solution of 16-C^{14}-estrone, 1 ml. of 0.9 per cent NaCl solution and various amounts of blood drawn from Subjects A, B, C and D. The tubes of Subjects C and D were centrifuged immediately while the tubes of Subjects A and B were allowed to stand for 30 minutes at room temperature (with occasional gentle mixing) prior to centrifugation. Plasma was removed quantitatively from each tube using a capillary pipette and measured carefully. The volume of plasma obtained was compared with the plasma volume expected on the basis of blood microhematocrit, taking into consideration the volumes of the radioestrone solution and saline solution added to each tube (see Table I).

Each plasma sample was extracted with chloroform. The extracts were evaporated to dryness and the residue was assayed for its radioactivity. The results were corrected on the basis of the expected plasma volume. As can be seen from Table I, 75.0 to 88.4 per cent of the radioactivity was calculated to be in the plasma of various blood samples, while the balance of the activity could be expected to accompany the red cells, *i.e.*, 25 to 11.6 per cent. It is to be noted that the results were not significantly different whether the blood samples were immediately centrifuged (Subjects C and D), or whether they were allowed to stand for 30 minutes.

Experiment No. 2

Nine centrifuge tubes were prepared, each one containing 0.2 ml. of radioestrone solution, 1 ml. of saline solution and 10 ml. of blood from Subject E. Three of them were placed in an incubator at 37° C., three were kept at room temperature (22° C.) and the remaining three maintained at 5° C. in a cold room. One tube of each group was centrifuged after standing for 30 minutes, another was allowed to stand for 120 minutes, while the last tube was kept 240 minutes prior to centrifugation. Following centrifugation, the various samples were treated as described in Experiment No. 1.

The results are shown in Table II and it can be seen that neither time nor temperature influenced the distribution of the radioactivity between plasma and red cells.

TABLE II

Effect of temperature and time on the in vitro distribution of 16-C^{14}-estrone between plasma and red cells of blood samples from Subject E (Experiment No. 2)

Experimental conditions						Per cent activity			
Temperature	Time	Blood vol.	Blood hemat.	Expected plasma volume + 1.2 ml.	Obtained plasma volume	In obtained plasma vol.	Expected in plasma	Found in RBC layer	Total recovery
	min.	*ml.*		*ml.*	*ml.*				%
	30	10	39	6.1 + 1.2 = 7.3	6.5	79.0	88.8	15.7	94.7
5° C.	120	10	39	6.1 + 1.2 = 7.3	6.5	78.5	88.2	19.7	98.2
	240	10	39	6.1 + 1.2 = 7.3	6.4	79.6	90.8	18.3	97.9
	30	10	39	6.1 + 1.2 = 7.3	6.5	78.0	87.3	18.5	97.5
22° C.	120	10	39	6.1 + 1.2 = 7.3	6.6	80.4	88.9	19.0	99.4
	240	10	39	6.1 + 1.2 = 7.3	6.4	77.8	88.8	18.2	96.0
	30	10	39	6.1 + 1.2 = 7.3	6.6	81.3	90.0	16.5	97.8
37° C.	120	10	39	6.1 + 1.2 = 7.3	6.4	78.0	88.9	15.7	93.7
	240	10	39	6.1 + 1.2 = 7.3	6.5	78.0	87.6	16.3	94.3

Experiment No. 3

In Experiment No. 1, following removal of plasma, the red cells of blood of the various samples were saved for the present experiment. The red cells of Sample 1 of Subject B and of Sample 2 of Subject C were washed with their volume of 0.9 per cent NaCl solution, while the red cells of Sample 1 of Subject D were washed with twice their volume saline; the red cells of Sample 1 of Subject C and Sample 2 of Subject D were washed with their volume of plasma (the plasma used for the washings came from the corresponding subjects). This procedure was carried out four times for the plasma washings and 10 times for the saline washings. Each of the washings was kept separately and extracted with chloroform. After these repeated washings, the red blood cells were precipitated and extracted with ethanol.

The radioactivity detected in the various extracts is shown in Figure 1, while the total recoveries are reported in Table III. When the logarithms of the washing values were plotted

TABLE III

Distribution coefficient of 16-C^{14}-estrone in the systems red blood cells and 0.9 per cent NaCl solution, or Krebs-Ringer solution, or plasma (Experiment No. 3)

	Washings			Distribution coefficient* Mean ± S.D.	Total recovery (in % of radio-estrone added)
	Type of washings	Volume	Number of washings		
Sample 1, Subj. D	0.9% NaCl	Twice RBC vol.	×10	0.197 ±0.010	104.56
Sample 2, Subj. C	0.9% NaCl	Once RBC vol.	×10	0.110 ±0.012	96.86
Sample 1, Subj. B	0.9% NaCl	Once RBC vol.	×10	0.094 ±0.009	93.56
Sample 1, Subj. C	Plasma	Once RBC vol.	× 4	3.18	93.57
Sample 2, Subj. D	Plasma	Once RBC vol.	× 4	3.25	104.11
Subj. E	Krebs-Ringer	Once RBC vol.	×10	0.098 ±0.008	98.05
Subj. F	Krebs-Ringer	Once RBC vol.	×10	0.099 ±0.011	97.62

* Washing No. 1 excluded.

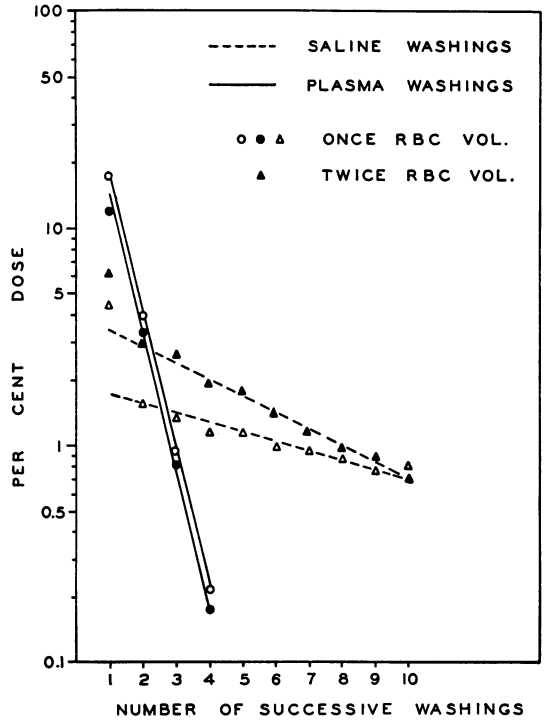

FIG. 1. RADIOACTIVITY IN SUCCESSIVE PLASMA WASHINGS OF TAGGED RBC, COMPARED WITH THAT OBTAINED IN SUCCESSIVE 0.9 PER CENT NaCl WASHINGS (EXPERIMENT No. 3)

(Figure 1) these values fell on a straight line with the exception of Washing No. 1. This is characteristic of the partition of a compound between two immiscible solvents. The distribution coefficient was calculated by two different methods. In the first method, the following formula was used:

$$K = \frac{\text{Wash No. 10}}{\text{RBC}} = \frac{\text{Wash No. 9}}{\text{RBC + Wash No. 10}} = \frac{\text{Wash No. 8}}{\text{RBC + Wash No. 10 + Wash No. 9}} = \cdots \text{ and so forth.}$$

In Method 2, the formula employed was:

$$K = \frac{\text{Wash No. 1}}{\text{Wash No. 2}} - 1 = \frac{\text{Wash No. 2}}{\text{Wash No. 3}} - 1 = \frac{\text{Wash No. 3}}{\text{Wash No. 4}} - 1 = \cdots \text{ and so forth.}$$

The results of these calculations are shown in Table III. Except for the distribution coefficients calculated on the basis of the activity in Wash No 1, the K values obtained were relatively constant. However, Method 1 gave a greater consistency than Method 2. This is probably due to the fact that small technical errors are reflected more strongly in the distribution coefficient calculated by the latter method; furthermore, no value could be obtained by this method when the activity in a washing was less than that obtained in the following wash.

In a similar experiment, where Krebs-Ringer solution was used for the washing of the red cells (Subjects E and F) instead of saline solution, identical results were obtained (Table III).

Experiment No. 4

Two centrifuge tubes were prepared containing 0.3 ml. of radioestrone, 9.7 ml. of saline solution and 10 ml. of red blood cells (without white cells) from Subject D. The tubes were shaken gently, but thoroughly, and were allowed to stand at room temperature for 30 minutes with occasional mixing. At the end of this time, the tubes were centrifuged and the saline solution was removed. The red cells were washed nine more times with 10 ml. of saline solution, and at the end of this procedure they were precipitated and extracted with ethanol.

The radioactivity remaining with the red cells represented 33.3 and 37.3 per cent of the estrone added. The values for the various saline washings, with the exception of the first one, fell on a straight line when plotted on semilogarithmic paper. The average distribution coefficients obtained by Method 1 were 0.090 ± 0.011 and 0.091 ± 0.002, while the total recoveries were 93.11 and 96.29 per cent of the radioestrone added.

Experiment No. 5

This was carried out to test the loading capacity of the red cells. The technique was similar to that described in the previous experiment, with the exception that in the first step the washed red cells from Subject D were loaded with nonradioactive estrone (5 to 100 μg.). Table IV summarizes the method and shows the results. It can be seen that the amount of radioactivity with the red cells was independent of the amount of nonradioactive estrone used in the first step of the experiment.

There was some doubt whether or not the total amount of nonradioactive steroid had been completely picked up by the red cells. In order to check on this point, the first step of the experiment was repeated and the saline washings were kept, pooled and extracted with chloroform. The chloroform was evaporated to dryness and a Kober reaction was performed on the residue. A known amount of estrone standard was added to the residue of Tube 6; only 80 per cent of the optical density expected from the amount of estrone standard added was obtained, the inhibition probably being due to the material resulting from slight hemolysis of red cells. Taking this value as standard, it was found that Tubes 1 to 5 contained, respectively, 11.2 13.0, 16.3, 13.6 and 18.0 per cent of the amount of nonradioactive estrone added. It would, therefore, appear that most of the estrone was picked up by the red cells in the first step of the experiment, but these cells were not overloaded since they were able to pick up the radioactive estrone in the second step of the test. In other words, the solubility of estrone in red cells is at least 82 μg. per 5 ml. (in Tube 5) or 6.08×10^{-5} M per L.

Experiment No. 6

A suspension of stroma (Subject E) in 1 : 10,000 acetic acid solution was prepared following the method described by Tischkoff, Robscheit-Robbins and Whipple (12). The stroma was separated from the liquid phase and then washed with saline solution in order to remove any trace of acetic acid solution.

A volume of 2.3 ml. of saline solution and 0.1 ml. of radioactive estrone was added to 2.4 ml. of stroma. After thorough mixing, the tube was centrifuged and the liquid phase was removed. Three more washings with 2.4 ml. of saline solution were carried out in a similar fashion. The washings were extracted with chloroform while the stroma residue was precipitated and extracted with ethanol as described for the red cells. The total recovery was 94.5 per cent and the stroma residue contained 65 per cent of the amount of the radioactivity added at the beginning of the experiment. The average distribution coefficient of estrone in the system stroma : saline solution was calculated to be 0.095 and was similar to that obtained for the system intact red cells : saline solution.

Experiment No. 7

A solution of hemoglobin in saline (Subject D) was prepared as follows: 30 ml. of 0.9 per cent NaCl solution was added to 10 ml. of washed red cells which had been separated from the white blood cells; the mixture was frozen and thawed

TABLE IV

Loading of RBC with nonisotopic estrone followed by tagging with 16-C^{14}-estrone (Experiment No. 5)

			Tube number					
			1	2	3	4	5	6
First step	a. Mix—centrifuge Saline discarded	Nonradioestrone (100 μg./ml.)	0.05 (5 μg.)	0.10 (10 μg.)	0.20 (20 μg.)	0.50 (50 μg.)	1.0 (100 μg.)	0
	b. Add 5 ml. saline Mix—centrifuge Saline discarded	0.9% saline solu. Washed red cells	9.95 5.0	9.90 5.0	9.80 5.0	9.50 5.0	9.0 5.0	10.0 5.0
Second step	a. Mix—centrifuge Saline removed (wash No. 1)	0.9% saline solu.	4.8	4.8	4.8	4.8	4.8	4.8
	b. Add 5 ml. saline Mix—centrifuge Saline removed (wash No. 2)	Radioestrone	0.2	0.2	0.2	0.2	0.2	0.2
	c. RBC precipitated							
	Results	Saline wash No. 1	12.6	11.6	9.6	11.2	11.3	10.9
		Saline wash No. 2	9.3	8.2	8.2	8.0	8.0	9.3
		RBC	73.0	75.8	67.5	79.5	63.0	75.4
		Total recovery	94.9	95.6	85.3	98.7	82.3	95.6

twice; it was then centrifuged and the aqueous phase constituted the hemoglobin preparation.

A centrifuge tube was prepared containing 0.1 ml. of radioestrone solution and 5 ml. hemoglobin solution; 5 ml. of washed red cells of Subject D was added and after thorough mixing and centrifugation, the liquid phase was removed. Three more hemoglobin solution washings were carried out. Each of these washings was extracted with chloroform and the red cells were precipitated with ethanol.

The total recovery was 97.4 per cent, the radioactivity left in the red cells was 40.3 per cent and that found in the successive hemoglobin solution washings was 22.2, 15.0, 11.0 and 8.9 per cent. The average distribution coefficient was calculated to be 0.247. It would appear that hemoglobin can remove radioestrone from the red cells faster than 0.9 per cent NaCl solution but not as efficiently as plasma.

Experiment No. 8

To a centrifuge tube containing 0.1 ml. of radioestrone solution and 7.9 ml. of saline solution, 4 ml. of washed red blood cells (Subject E) freed of white cells was added and mixed. The mixture was frozen and thawed twice. It was then centrifuged and the liquid phase was removed and extracted with chloroform. The red cell ghosts, approximately 0.6 ml. in volume, were extracted twice with ethanol.

The liquid phase contained 67.8 per cent of the radioactivity while the solid phase (red cell ghosts) contained 31.5 per cent. It is certain that this latter phase was contaminated with a certain amount of the upper phase. However, if the solid volume had been made entirely of the liquid phase it would have a maximal activity of 3.5 per cent. The red cell ghosts, therefore, had retained a significant amount of radiosteroid. It must also be considered that the liquid phase consisted not only of the red cell fluid but also of 8 ml. of saline solution, representing twice the initial red cell volume. The saline solution of the first washing was found to contain amounts of activity relatively greater than those which could be predicted from the distribution coefficient of the system considered. It follows that hemoglobin was responsible for only a fraction of the 67.8 per cent of the radioactivity detected in the liquid phase.

DISCUSSION

In Experiment No. 1, the calculation of the distribution of radioestrone between plasma and red cells of a given sample is based on the assumption that the value of the microhematocrit gives the real volume of the erythrocytes. When we compared the values of hematocrits obtained by our micro-method and those found with the Wintrobe method (2,080 G for 30 minutes), it was observed that the latter technique gave results 2.4 to 2.9 per cent higher. Since it is generally admitted that the amount of plasma trapped in packed red cells is between 2 and 2.8 per cent of the cell volume obtained by the Wintrobe technique (13, 14), it seems that our assumption was correct. However, theoretically, the percentage of activity calculated to be with the red cells is still probably slightly overestimated.

One could expect that a similar distribution of estrone between plasma and red cells would take place *in vivo;* indeed, we found that following intravenous administration of 16-C^{14}-estrone to three normal adults, the red cells contained 7.9 to 18.7 per cent of the total free activity detected in various blood samples (9). However, since the *in vitro* distribution was immediate and not influenced by temperature in the range of 5° to 37° C. (Experiment Nos. 1 and 2), it is impossible to decide whether this distribution takes place before or after the withdrawal of the blood.

The significance of the *in vivo* studies is limited also by the fact that estrone undergoes extensive metabolism almost immediately after administration and, therefore, the nature of the free steroids of plasma is not necessarily the same as that of the free steroids of red cells.

It is of interest that the red blood cells behaved (Experiment Nos. 3 and 4) as an ordinary solvent for 16-C^{14}-estrone as demonstrated by the existence of a distribution coefficient for the steroid in the system used. It is not surprising that the coefficient would be greater with plasma than saline solution, since it is known that some of the plasma proteins can increase the solubility of steroids (15, 16).

Experiment No. 5 was carried out in order to

study estrone loading of red cells. The results must be considered with some reservation. As pointed out previously, we were not certain that the total amount of nonisotopic estrone was taken up by the red cells. It was found impossible to measure estrone chemically in an extract of red cells because of the presence of interfering chromogens. It was necessary to measure the estrone content of the saline washings and to assume that the amount of steroid which was not in these washings was with the red cells; however, this latter chemical determination was also partly impeded by chromogen interference.

The data presented do not permit us to decide whether estrone was in solution in the intracellular fluid of the red cells or whether it was absorbed on the surface of the cells. The latter possibility seems to be the more probable since an active transfer through a cell membrane is usually influenced by temperature variation; this was not found to be true in Experiment No. 2. Furthermore, estrone had a distribution coefficient in the system saline solution: red cells similar to that found in the system saline solution: stroma (Experiment No. 6) suggesting that the adsorption of radioestrone on stroma could account for the amount of activity associated with the red cells. On the other hand, the addition of hemoglobin to 0.9 per cent NaCl solution increased the distribution coefficient of estrone, showing that hemoglobin itself increases the solubility of the steroid (Experiment No. 7). This might explain why only one-third of the radioestrone was recovered from the red cell ghosts (Experiment No. 8) and, hence, does not necessarily invalidate the theory of surface adsorption of the steroid.

In distribution studies, the compound distributed must not react with the solvents. It has been shown that 4 to 5 per cent of estrone added to whole blood is transformed into estradiol (14). The enzymatic system which produces biological activation requires the presence of glucose, is not destroyed on hemolysis of the red cell and the optimum pH range is 6.6 to 8.2. It is destroyed by heating for one hour at 63° C., by 20 per cent ethanol and by a reaction pH of 4.7 (5). Our experience has been similar (17). However, as pointed out by Bischoff and associates (5), prolongation of the incubation period increases the biological activation and we have observed that the formation of estradiol is insignificant when the period of incubation is 30 minutes or less.

Since 12 to 25 per cent of estrone appears to be associated with the red cells, then the red cells must play a rather important role in the transport of the hormone; the red cells have been found also to produce biological activation of estrone by production of estradiol (5, 14) while plasma alone does not cause such an activation (17). These various findings stress the significance of the study of estrone binding to red blood cells of man. In addition, red cells tagged with radioestrone have been observed to be an interesting tool for the study of the relative binding capacity of various plasma protein fractions (18).

SUMMARY

When 16-C^{14}-estrone was added to blood samples of normal individuals, 75.0 to 88.4 per cent of the radioactivity was recovered in the plasma; the balance of the activity accompanied the red cells. Neither the length of the incubation (0 to 4 hours) nor temperature (5, 22 and 37°C.) appeared to influence this distribution.

The activity accompanying the red cells was slowly removed by successive washings with 0.9 per cent NaCl solution, the average distribution coefficient of 16-C^{14}-estrone for this system being 0.094 to 0.125. The activity was removed much faster by plasma, the average coefficient being 3.09 to 3.29.

It was calculated that the solubility of estrone in red cells was at least 6.08×10^{-5} M per L. and probably greater since overloading was not achieved. The average distribution coefficient of radioestrone between 0.9 per cent NaCl solution and red cell stroma was 0.095, while that between a saline solution of 25 per cent hemoglobin and red cells was 0.247. The suggestion that the phenomena studied were related to adsorption of estrone on the red cell surface was discussed.

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