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Research Article

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IN VITRO INTERCONVERSION OF 16-C^{14}-ESTRONE AND 16-C^{14}-ESTRADIOL-17β BY ERYTHROCYTES FROM NORMAL SUBJECTS AND FROM SUBJECTS WITH A DEFICIENCY OF RED CELL GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY *

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Werthessen and his collaborators (2–4) reported that incubation of estrone in blood produced the destruction of the 17-ketone function of the steroid, accompanied by a loss of biological activity. On the other hand, Bischoff and associates (5, 6) incubated the red blood cells of rabbit or man with estrone and observed a marked increase of the biological activity of the added steroid. The discrepancy in the two sets of data was ascribed at the time to differences either in assay techniques or in experimental conditions. A similar increase in biological activity was reported by Repke and Markwardt (7), who used red cells of the guinea pig, rabbit, and man. It now appears that species differences were the cause of this discrepancy. In most of these experiments, Werthessen and co-workers (3, 4) used bovine blood, and Axelrod and Werthessen (8) have found that in this species estrone is converted to the extent of 88 per cent into estradiol-17α. The investigations of Bischoff and associates (5, 6) were carried out with rabbit and human red cells, and these authors (9) have shown that the main reaction product resulting from estrone incubation in their system was estradiol; the latter report, however, failed to mention whether the steroid detected was the α- or β-isomer, but there are reasons to believe that reaction product was estradiol-17β. This isomer is biologically more active than estrone or estradiol-17α; hence the difference in the results reported by Werthessen and co-workers (3, 4) and Bischoff and associates (5, 6). In a study of the stereospecificity of the activity of estradiol dehydrogenases in the erythrocytes of man and of various animals, Portius and Repke (10) showed that an estradiol-17β dehydrogenase was present in the red cells of the 15 species investigated, whereas an estradiol-17α dehydrogenase was found only in ruminants such as cow, sheep, and goat. The same authors (10) reported that the activity of the β-enzyme was the highest in the rat and the lowest in man.

Bischoff and associates (6) have investigated some of the conditions influencing the "activation" of estrone, but the degree of transformation of the steroid was determined by bio-assay. Repke and Markwardt (7) have also studied this problem using the Allen-Doisy test as an index of "activation" of estrone, and have found that neither the succinic dehydrogenase systems nor the enzyme systems involved in red cell glycolysis were able to transform estrone to estradiol-17β. In another paper, the same workers (11) reported that preparations of glucose-6-phosphate dehydrogenase of either guinea pig red cells or yeast (12) could in the presence of glucose-6-phosphate and TPN reduce estrone to estradiol-17β. Bischoff, Torres, and Lopez (13) confirmed some of these findings. More recently, Portius and Repke (14) have studied the properties of estradiol-17β dehydrogenase of rat erythrocytes and have attempted to purify the enzyme.

It is the purpose of the present paper to report the results of our personal experience with the interconversion of 16-C^{14}-estrone and 16-C^{14}-estradiol-17β by the red blood cells of normal subjects and individuals who have a hereditary alteration of glucose-6-phosphate dehydrogenase activity in their erythrocytes (15).

* This work was made possible by grants-in-aid A-180 (C10), H-3995, and B-1612 from the National Institute of Health, U. S. Public Health Service. The data were presented at the First International Congress of Endocrinology, Copenhagen, 1960 (1).

METHODS

Purity and standardization of the radioactive steroids. The 16-C^{14}-estrone and 16-C^{14}-estradiol-17β were purchased from Charles E. Frosst Company, Montreal, Canada. Their specific activities were 0.729 and 0.734 mc per mM. Their purity was tested as previously described (16) and was found to be 96 to 98 per cent.

The steroids were dissolved in 100 per cent ethanol. This concentrated solution was diluted with 0.9 per cent NaCl solution, the final mixture containing 5 per cent of ethanol.

Basic experimental system and its variations in experiments with nonhemolyzed erythrocytes. Except for one experiment where the effect of defibrination, citration, and decalcification was studied, all the blood samples were heparinized. The samples were centrifuged immediately at 2,000 rpm for 30 minutes. The plasma and buffy coat were removed as completely as possible, and the red cells were washed twice with three volumes of 0.9 per cent NaCl solution.

The basic system for 16-C^{14}-estrone as starting material consisted of 0.5 ml of washed red blood cells, 0.5 ml of 0.9 per cent NaCl solution, 0.1 ml of 5 per cent glucose solution, and 0.2 ml of estrone solution (approximately 4,000 cpm or 1.3 μg of steroid). In the basic system for 16-C^{14}-estradiol-17β, the glucose solution was replaced by 0.1 ml of 0.9 per cent NaCl solution.

The mixture was incubated at 37°C for 24 hours. On several occasions, the incubation mixtures were checked for the presence of microorganisms, but the cultures were negative. At the end of the incubation, the steroids were extracted twice with 4 ml of ethanol and once with 4 ml of a 1:1 (vol/vol) mixture of ethanol and butanol. The pooled extracts were then evaporated to dryness and the residue was mixed with 3 ml of distilled water. The aqueous phase was extracted five times with 6 ml of chloroform, and the combined chloroform was evaporated to dryness. The residue was applied on Whatman paper No. 2 and chromatographed as previously described (16). The paper chromatogram was radioautographed (16), the area corresponding to estrone and estradiol-17β was eluted with ethanol, and the amount of radioactivity present in the eluates was determined.

When variations were made in the basic system, the final volume of 1.3 ml was maintained by an appropriate change in the volume of saline solution added. The only exception was in the experiments on the effect of the amount of red cells used (with 2 and 3 ml of red cells, the final volumes were 2.3 and 3.3 ml, respectively). In the experiments on the effect of pH, 0.5 ml of buffer solution replaced the 0.5 ml of saline solution. In other experiments, the saline solution was replaced by 0.5 ml of 1, 0.5, 0.25, and 0.12 per cent citric acid in saline solution in order to bring the initial pH of the mixture to 5.2, 5.8, 6.3, and 6.6, respectively; 0.5 ml of 0.75, 0.5, and 0.25 per cent Na_2CO_3 in saline solution was used for obtaining an initial pH of 9.2, 8.7, and 7.9. In the inhibition experiments, methylene blue and sodium fluoride were dissolved in saline solution, and 0.5 ml of these mixtures replaced the regular saline solution.

Basic experimental system in experiments with hemolyzed erythrocytes. A volume of 0.5 ml of washed red cells was transferred by pipet into a test tube which was placed in a freezer at −50°C for 30 minutes. After thawing, various solutions were added as indicated in each corresponding Table. Finally, the estrogen solution was added and the final volume was brought to 2.1 ml with 0.9 per cent NaCl solution. The samples were then treated as described for the intact red cells.

In experiments in which red cell ghosts were removed, this procedure was followed: After the freezing of a known volume of red cells, saline solution was added (1.5 the volume of red cells); the mixture was centrifuged at 28,000 × G, at 5°C for 60 minutes; 1.25 ml of the aqueous solution was used for these experiments.

For the $(NH_4)_2SO_4$ precipitation, the red cell ghosts were removed, and to 4 ml of the red cell hemolysate an equal volume of either 50 per cent, 75 per cent, or 100 per cent $(NH_4)_2SO_4$ was added, giving a final concentration of 25, 37.5, and 50 per cent, respectively. The mixture was centrifuged at 28,000 × G at 5°C for 60 minutes. Two ml of supernatant fluid (corresponding to 0.5 ml of initial red cells), 0.3 ml of 0.01 M TPN, and 0.3 ml of 16-C^{14}-estradiol-17β were incubated and then treated as described above. Each of the precipitates was dissolved in 2 ml of saline; 0.5 ml of this solution, 0.3 ml of 0.01 M TPN, 0.3 ml of 16-C^{14}-estradiol-17β, and 1.5 ml of saline were also incubated.

The DEAE (diethylaminoethyl cellulose) used for column fractionation was washed with 1N NaOH, 0.9 per cent NaCl solution, 95 per cent ethanol, and then was dried. Prior to use, 0.9 per cent NaCl solution was added to DEAE and the pH adjusted to 7.0. A column of 1.8 cm in diameter and 8.0 cm in length was prepared. Prior to applying the red cell hemolysate, the column was washed with 50 ml of cold 0.005 M NaCl. Elution of the column was then carried out by adding increasing concentrations of NaCl solution and by collecting 10 ml fractions. A 4-ml sample of each of these fractions, 0.3 ml of 0.01 M TPN, and 0.3 ml of 16-C^{14}-estradiol-17β were incubated and then treated as described above.

Recovery. The radioactivity recovered after extraction of the basic experimental system and prior to paper chromatography represented 94 to 103 per cent of the radioactivity originally added to the system. In all the experiments, the sum of the activities eluted from the areas of the paper chromatograms corresponding to estrone and estradiol-17β represented 85 to 92 per cent of the radioactivity added at the beginning of the experiment.

When the extract of more than 0.5 ml of red cells was chromatographed, the increased amount of nonsteroidal impurities was found to produce a displacement of the steroids on the paper chromatogram. For this reason, in experiments in which the amount of red cells was 1, 2, or 3 ml, extract samples corresponding to 0.5 ml of cells were chromatographed separately.

Experimental subjects. In all the experiments, the blood samples were collected from normal young adult subjects. In order to study the possibility of daily variations, blood samples were obtained from five normal females 23 to 32 years of age, at various times of the menstrual cycle, and from four normal males 28 to 37 years of age at weekly intervals.

Samples were collected from ten individuals with a deficiency of red cell glucose-6-phosphate dehydrogenase activity; six were American negroes with a 90 per cent reduction of enzyme activity, one was a Sephardic Jew with a 95 to 98 per cent decrease in enzyme activity, and three were Caucasians in whom no activity could be determined in the crude hemolysate. In the last group, the abnormality was associated with a congenital nonspherocytic hemolytic anemia.

Activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase in the red cells was determined by a modification of the method of Glock and McLean (17, 18). The normal range for glucose-6-phosphate dehydrogenase was found to be 135 to 186 U per 100 ml of erythrocytes with an average of 156 (18).

RESULTS WITH NONHEMOLYZED RED BLOOD CELLS

1. Characterization of the products of the reaction. The reaction product resulting from the incubation of 16-C^{14}-estrone with red cells was chromatographed on paper in two different systems of solvents (isoöctane : toluene : methanol : water, 25 : 75 : 80 : 20; and benzene : Skelly C : methanol : water, 40 : 60 : 70 : 30) and found to have an R_F value identical with that of estradiol-17β in both systems. The material resulting from several incubations was eluted from the paper chromatogram. An exact amount of pure estradiol-17β (25 mg) was added to the pooled eluates (48,200 cpm), and on three successive crystallizations, the specific activity was constant as shown in Table I. The remaining mother solutions and crystalline material were pooled. Methylation was carried out as described by Brown (19), and the specific activity of the methylated compound on two successive crystallizations is shown in Table I. A sample of the pooled mother solutions of the two preceding crystallizations was chromatographed as described by Brown (19), and the amount of Kober chromogen and of radioactivity was determined in the estradiol fraction. From the amount of estradiol present as determined by the Kober reaction, the specific activity was calculated to be 3,080 cpm per mg.

By similar criteria, the product resulting from the incubation of 16-C^{14}-estradiol-17β with red blood cells was found to be radioactive estrone (see Table I).

Some of the radioautograms showed that a certain amount of radioactivity (about 4.8 per cent of the total activity) remained on the starting line of the papergram. It is thought to be due to nonspecific adsorption of the radiocompounds on the area of the paper where the extract was applied. On occasion a small quantity of radioactivity was detected also at the solvent front of the paper; such radioactivity, however, was closely related to nonpolar impurities of the extracts that were detectable by their fluorescence in ultraviolet light and by their absorption of this light when a fluorescence scanner was used (20). In view of our experience with nonspecific adsorption of a radiosteroid by some components of extracts of red cells, it is thought that the radioactivity found at the solvent front is probably not a metabolite of estrone or estradiol-17β resulting from the red cell incubation.

2. Specificity of the red cells. When the red cells were replaced by an equivalent volume of either saline or plasma, or plasma protein frac-

TABLE I

Specific activity of compound resulting from the incubation of human intact erythrocytes with 16-C^{14}-estrone or 16-C^{14}-estradiol-17β

Incubation of RBC	Resulting compound	Crystallization			
		Initial	First	Second	Third
With 16-C^{14}-estrone	Radioestradiol-17β	2,940	3,100	2,910	3,030
	Methyl ether *		2,890	2,960	
With 16-C^{14}-estradiol-17β	Radioestrone	2,440	2,330	2,390	2,500
	Methyl ether *		2,490	2,410	

* Corrected for the difference in the molecular weight of the methyl ether.

TABLE II

Effect of defibrination, citration, decalcification, and heparinization on the conversion of 16-C^{14}-estrone to 16-C^{14}-estradiol-17β

	Defibrination[1]	Citration[2]	Decalcification[3]	Heparinization[4]
Whole blood [5]	7.0	5.0	6.6	5.3
Whole blood [5] + 0.1 ml glucose	16.5	12.0	17.1	19.0
0.5 ml unwashed RBC + 0.1 ml glucose + 0.85 ml saline	24.0	29.0	32.0	31.0
0.5 ml washed RBC + 0.1 ml glucose + 0.85 ml saline	34.0	32.4	39.5	37.3

[1] 5 glass beads added to 10 ml blood. Swirled 20 minutes.
[2] 1 ml 3.8 per cent Na Citrate per 10 ml blood.
[3] 1 ml 0.05 per cent EDTA per 10 ml blood.
[4] 0.1 ml heparin per 10 ml blood.
[5] Blood volumes adjusted to correspond to 0.5 ml of RBC. If necessary, normal saline was added to give a final volume of 1.55 ml.

tions,[1] the transformation was negligible (0.2 to 0.8 per cent).

Since the washed red cell preparations were not completely freed of white cells, one might wonder whether the white cells were the active elements in the metabolism of estrone. In order to study this possibility, a solution containing 2,700 white cells and 18,000 red cells per mm^3 was incubated with glucose and estrone as usual. The results were compared with those obtained with a solution containing 300 white cells and 5,320,000 red cells per mm^3. The reaction mixture containing predominantly white cells produced a 5.8 per cent transformation of estrone to estradiol-17β, whereas the second one gave a 28.5 per cent transformation under the same reaction conditions.

3. Other enzymatic activities displayed by red blood cells. When 4-C^{14}-testosterone replaced estradiol-17β, a 65 per cent transformation to C^{14}-Δ^4-androstene-3,17-dione was observed.

4. Effect of defibrination, citration, and decalcification as compared with heparinization. The conversion of estrone to estradiol-17β was not influenced by the technique of anticoagulation employed (Table II). The percentage conversion was smaller when unwashed red cells were used and even smaller with whole blood.

5. Glucose requirement. As can be seen in Figure 1, glucose was necessary for the transformation of estrone to estradiol-17β, maximum transformation being obtained with concentration of glucose greater than 3.5 mg per 1 ml of mixture and a 2 to 4 per cent transformation being achieved when no glucose was added. At optimal concentrations, fructose was as effective as glucose but sucrose, galactose, ribose, xylose, and glucose-6-phosphate produced a transformation similar to that when no glucose was added. In similar experiments, but with the saline solution being replaced by plasma and no glucose added, a 5 to 8 per cent transformation was attained, whereas the

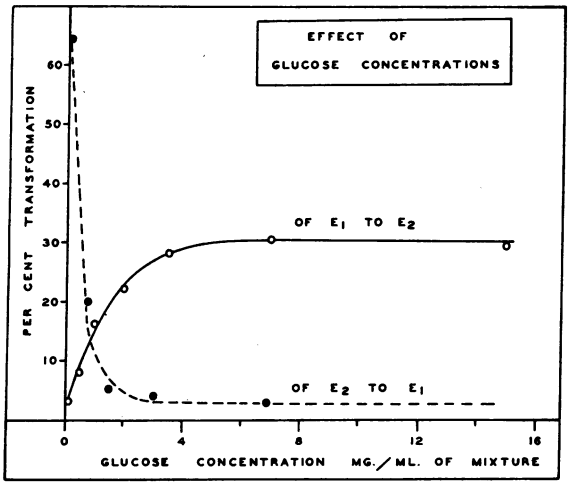

FIG. 1. EFFECT OF GLUCOSE CONCENTRATIONS ON THE INTERCONVERSION OF 16-C^{14}-ESTRONE (E_1) AND 16-C^{14}-ESTRADIOL-17β (E_2) BY HUMAN INTACT RED CELLS.

[1] The plasma protein fractions were supplied by Dr. Harry N. Antoniades, of Protein Foundation in Boston, Mass., and were prepared as described elsewhere (21).

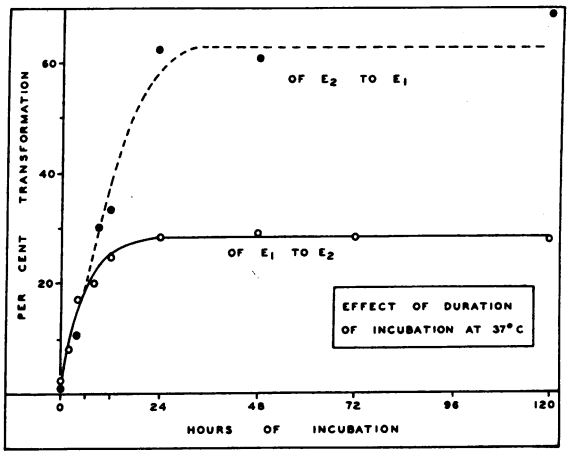

FIG. 2. EFFECT OF THE LENGTH OF INCUBATION AT 37°C ON THE INTERCONVERSION OF 16-C^{14}-ESTRONE (E_1) AND 16-C^{14}-ESTRADIOL-17β (E_2).

addition of glucose produced a 19.0 per cent transformation (Table II).

When estradiol-17β was used as substrate, its transformation was inhibited by glucose (Figure 1).

6. Effect of duration of incubation at 37° C. A 24-hour incubation was found to produce the maximum transformation (Figure 2). Shorter periods of incubation resulted in smaller percentages of transformation.

7. Effect of temperature of incubation. As shown in Figure 3, the maximal transformation

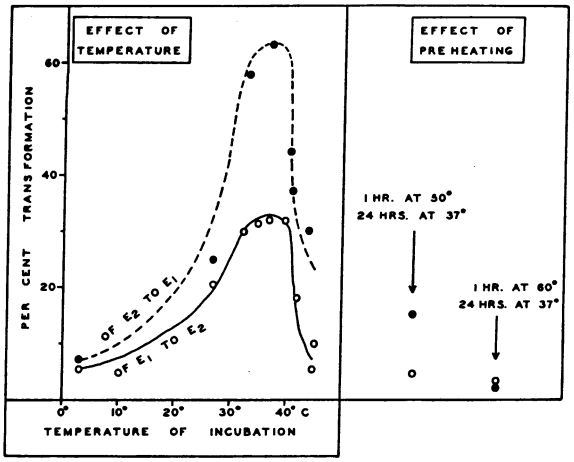

FIG. 3. EFFECT OF THE TEMPERATURE AT WHICH THE INCUBATION IS CARRIED OUT FOR 24 HOURS ON THE INTERCONVERSION OF 16-C^{14}-ESTRONE (E_1) AND 16-C^{14}-ESTRADIOL-17β (E_2). Preheating of the red cells at 60°C for one hour inhibits completely the interconversion.

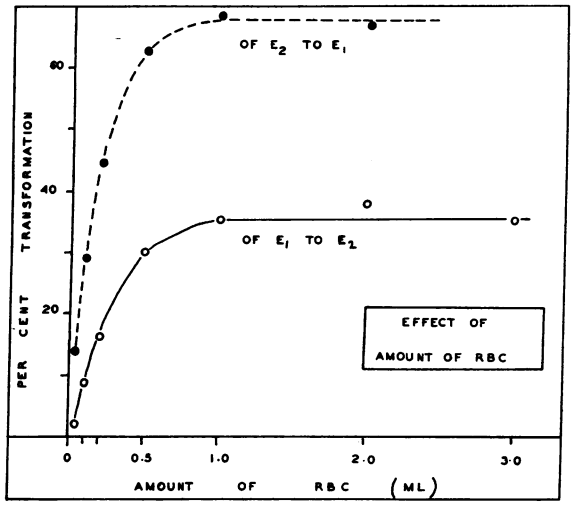

FIG. 4. EFFECT OF THE AMOUNT OF RED BLOOD CELLS PRESENT IN THE INCUBATION MIXTURE ON THE INTERCONVERSION OF 16-C^{14}-ESTRONE (E_1) AND 16-C^{14}-ESTRADIOL-17β (E_2).

was achieved by a 24-hour incubation at 35 to 40° C. The rapid fall in transformation with temperature above 40° C is probably related to partial hemolysis of the red cells. It is not possible to tell presently whether the inhibition of the transformation that related to a one-hour exposure of the red blood cells to a temperature of 50 to 60° C is due to destruction of the enzyme systems, or whether it is due to the complete hemolysis of the red cells.

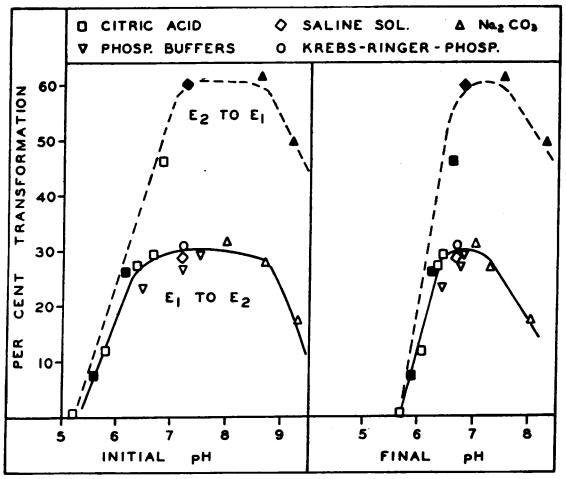

FIG. 5. EFFECT OF pH OF INCUBATION MIXTURE ON THE INTERCONVERSION OF 16-C^{14}-ESTRONE (E_1) AND 16-C^{14}-ESTRADIOL-17β (E_2) BY HUMAN ERYTHROCYTES.

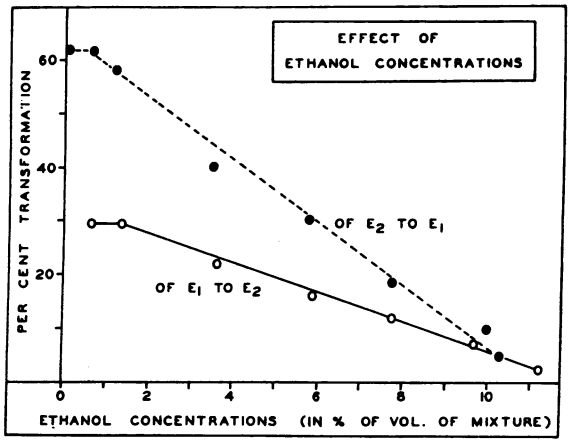

FIG. 6. EFFECT OF ETHANOL CONCENTRATIONS ON THE INTERCONVERSION OF 16-C^{14}-ESTRONE (E_1) AND 16-C^{14}-ESTRADIOL-17β (E_2) BY HUMAN RED CELLS.

8. Effect of amount of red cells. The amount of red cells used in the incubation was found to have an influence on the degree of conversion as seen in Figure 4. Maximal transformation was obtained with approximately 1 ml of red cells under the conditions of the reaction.

9. Effect of pH. It was observed that 0.1 M phosphate buffer (Clark and Sub's standard mixture of 50 ml 0.2 M KH_2PO_4 plus variable volumes of 0.2 M NaOH, diluted to 100 ml) or

TABLE III

Steroid interconversion by red cells of normal subjects

Subjects	% Steroid transformation	
	E_1 to E_2*	E_2 to E_1
	Mean Range	*Mean Range*
Five Normal Females Menstrual cycle days		
2–5	35 (32–28)	54 (50–58)
9–12	34.5 (26–42)	58 (49–70)
16–19	29.5 (29–32)	57 (50–65)
23–26	31.1 (26–38)	53 (49–60)
Four Normal Males Experimental day		
1	35.5 (28–40)	61 (56–68)
8	35.5 (31–42)	57.5 (53–61)
15	30.5 (27–33)	54 (49–61)

* E_1 = estrone; E_2 = estradiol-17β.

TABLE IV

Enzyme activity and steroid interconversion by the red cells of subjects with a congenital deficiency of glucose-6-phosphate dehydrogenase in their erythrocytes *

Subject	Sex	Age	RBC enzymes		% Steroid transformation	
			G-6-PD†	6-P-GD†	E_1 to E_2	E_2 to E_1
		yrs				
American Negroes with primaquine-sensitive RBC						
F. Mc Gr.	F	42	29	173	12.9	58.2
L. Mc Ne.	M	45	23	187	5.0	51.5
Ja. Mc Gr.	M	11	15	146	9.1	62.2
Sy. Mc Gr.	M	9	13	122	11.8	76.2
Sh. Mc Gr.	F	2	15	254	9.0	
M. Bl	F	32	38	254	10.2	68.1
Sephardic Jew with primaquine-sensitive RBC						
R. Da.	M	32	2	165	8.9	65.4
Patients with congenital non-spherocytic hemolytic anemia and family members						
D. Iy.	M	56	0	150	7.5	60.6
His Wife			162	143	30.4	59.8
A. Ki.	M	11	0	153	8.1	57.0
His Father			132	167	34.6	70.1
His Mother			77	152	22.0	62.4
E. Ro.	M	6	0	172	7.6	63.3
His Father			146	147	28.8	65.1
His Mother			115	148	20.2	61.0

* Abbreviations: G-6-PD = glucose-6-phosphate dehydrogenase; 6-P-GD = 6-phosphate-gluconic dehydrogenase; E_1 = estrone; E_2 = estradiol-17β.

† Activity is expressed as units of enzyme per 100 ml of RBC.

Krebs-Ringer-phosphate solution (22) were inadequate in the amounts used to produce sufficient buffering capacity until the end of the incubation. The effect of initial pH on the interconversion of estrone and estradiol-17β is shown in Figure 5. The final pH values differed from the initial values by as much as 1.0 pH unit for the highest pH to 0.25 pH units at the lowest pH tested. This could be explained by either the formation of lactate during the incubation or the effect of hemoglobin buffering capacity, or both.

With the more concentrated solutions of citric acid, a great deal of hemolysis occurred which could in fact account for the smaller conversions.

10. Inhibition by ethanol, sodium fluoride, and methylene blue. Ethanol produced marked inhibition of the transformation (Figure 6) when it was present in concentrations of more than 2 per cent (per volume). Inhibition by sodium fluoride was demonstrable with concentrations greater than 5×10^{-3}M.

Methylene blue appeared to have also some inhibitory action at concentrations greater than 0.01 mg per 1 ml of mixture.

11. Serial determinations in normal females and males. The conversion of estrone to estradiol-17β and the reversal of the reaction are shown in Table III. It can be seen that the percentage of transformation in males was somewhat variable from one week to the next one, but the results remained in the fairly narrow limit of 26 to 43 per cent transformation for estrone and 49 to 70 per cent for estradiol-17β. The results obtained in normal females varied similarly, and the menstrual cycle did not appear to influence significantly the percentage of transformation.

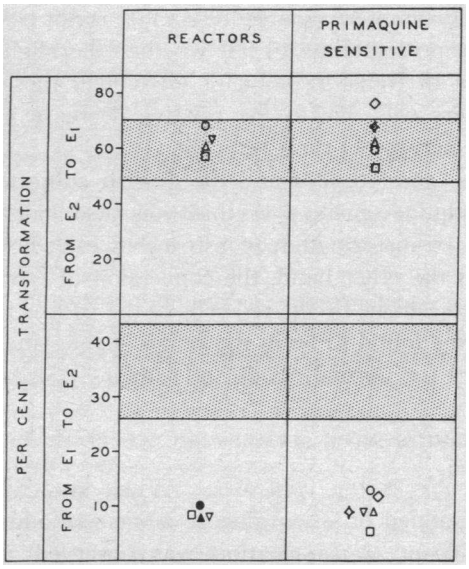

FIG. 7. *IN VITRO* INTERCONVERSION OF ESTRONE AND ESTRADIOL-17β (E_2) BY THE RED CELLS OF SUBJECTS WITH CONGENITAL DEFICIENCY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN THEIR ERYTHROCYTES. The shaded areas represent the range of transformation obtained with the red cells of normal individuals.

12. Subjects with a deficiency of the red cell glucose-6-phosphate dehydrogenase and their relatives. The results are shown in Table IV. In the patients with congenital nonspherocytic hemolytic anemia, the percentage of transformation of estrone to estradiol-17β was markedly reduced. It was normal in the fathers of the patients and the wife of one, but it was at the lower limit of the normal range in the two tested mothers of patients. Table IV shows that these two mothers had a somewhat decreased glucose-6-phosphate dehydrogenase activity in their red cells and are usually referred to

TABLE V

Effect of addition of various cofactors on steroid interconversion by hemolyzed red cells of normal subjects *

Solutions added (ml)					% Steroid transformation	
Glucose (5%)	G-6-P (0.5 M)	TPN (0.01 M)	TPNH (0.01 M)	DPN (0.01 M)	E_1 to E_2	E_2 to E_1
0.1					2.6 (5)	2.1 (5)
					3.2 (6)	4.1 (4)
		0.3			4.4 (4)	70.2 (7)
	0.1				4.1 (4)	
	0.1	0.3			35.1 (16)	2.4 (2)
			0.3		34.3 (3)	2.9 (2)
				0.3	2.2 (3)	3.1 (3)

* The results are the mean values of a number of experiments as indicated in parentheses. Abbreviations: G-6-P = glucose-6-phosphate; E_1 = estrone; E_2 = estradiol-17β.

as "female intermediates" (23). It is not possible at the present time to tell whether the slight decrease in transformation of estrone in these two mothers was due to the relative decrease in enzyme activity of their red cells.

The six Negroes and the Jewish subject with primaquine-sensitive erythrocytes also had a reduced transformation of estrone to estradiol-17β.

On the other hand, the conversion of estradiol-17β to estrone by the red cells of the subjects studied was found to be in the range of the values obtained for normal males and females (Figure 7).

RESULTS WITH HEMOLYZED RED BLOOD CELLS

1. Necessary cofactors. When no cofactors were added or when glucose alone was added, no significant transformation was observed (Table V). The transformation of estradiol-17β to estrone required TPN; DPN could not replace TPN. The reverse reaction required either TPNH or TPN and glucose-6-phosphate.

2. Effect of removal of red cell ghosts. In the absence of red cell ghosts, results similar to those shown in Table V were obtained.

3. $(NH_4)_2SO_4$ precipitation. With 25 per cent $(NH_4)_2SO_4$ the enzymatic activity was found to be entirely in the supernatant fluids, whereas with 37.5 per cent the activity was in both the supernatant fluids and the precipitates. With 50 per cent, only the precipitates transformed estradiol-17β to estrone.

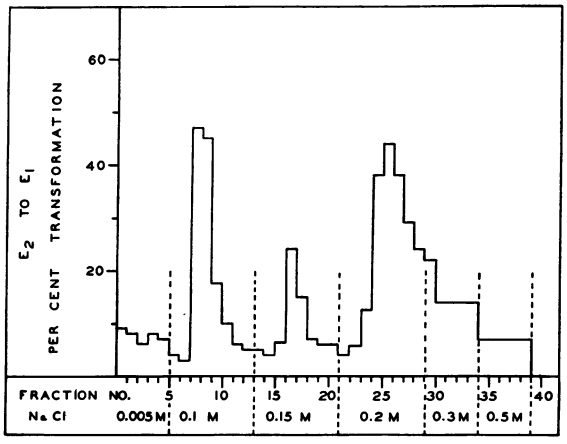

FIG. 8. DEAE COLUMN FRACTIONATION OF THE ESTRADIOL-17β (E_2) DEHYDROGENASE ACTIVITY IN RED CELL HEMOLYSATE. The activity is expressed as percentage of transformation of estradiol-17β (E_2) to estrone.

4. DEAE column fractionation. As shown in Figure 8, three peaks of enzymatic activity were obtained. The red cells of four different subjects showed similar peaks; however, the height of each was found to be variable.

DISCUSSION

The results show that intact red blood cells of man can convert 16-C^{14}-estrone to 16-C^{14}-estradiol-17β, confirming the data of Bischoff and his collaborators (5, 9). These authors, however, did not specify the stereoisomeric position of the 17-hydroxyl group. It is most interesting to note the species differences reported by Axelrod and Werthessen (8), who have found that estradiol-17α was the major conversion product of 16-C^{14}-estrone in pregnant cow blood. In an analogous manner, Lindner (24) has observed that Δ^4-androstene-3,17-dione was transformed into epitestosterone by bovine blood, and we have found that the same substrate gave rise to testosterone when incubated with red blood cells of man.

The reversibility of the *in vitro* conversion of estrone to estradiol-17β has been demonstrated by the data presented above, as well as by other investigators (10–12, 14). The evidence suggests that the reaction is enzymatically mediated. Bischoff and associates (6) have suggested the name of "estronase" for the enzyme. "Estradiol-17β dehydrogenase" would be more appropriate, as proposed by Portius and Repke (14). In addition, it is quite possible that the interconversion of Δ^4-andostene-3,17-dione and testosterone that we have observed may also be mediated by the same enzyme, a 17β-hydroxysteroid dehydrogenase.

Our study of the factors influencing the transformation of estrone to estradiol-17β by intact human red blood cells confirms the data published by Repke and his collaborators (12, 14), who used mainly rat erythrocytes and a yeast preparation. These results are also in agreement with the earlier work of Bischoff and associates (6). An important difference with the latter data is the fact that in our experiments hemolysis of the red cells arrested the transformation. If glucose-6-phosphate and TPN were added, however, estrone was converted to estradiol-17β, whereas the addition of TPN only to hemolyzed cells permitted the reverse reaction. Hemolysis of red cells is

known to destroy hexokinase and TPN activity, and it is not surprising that the addition of glucose alone could not affect the transformation of estrone. The interconversion was found to be inhibited by very small concentrations of ethanol, but the effect of sodium fluoride or methylene blue was not so marked. One might wonder whether the inhibition produced by these substances was not related to a certain degree of hemolysis of the red cells. The same comment could apply to the effect of preheating the red cells.

The need of glucose for the transformation of estrone to estradiol-17β by intact red cells of normal man has suggested that the reaction is related to the glycolysis that takes place in the cells (6). Since the reaction involves a hydrogen transfer, it was reasonable to assume that a hydrogen donor was required. Repke and Markwardt have shown that the glucose-6-phosphate dehydrogenase and TPN system was involved in the steroid conversion (7).

In subjects with a deficiency of glucose-6-phosphate dehydrogenase, the transformation of glucose-6-phosphate to 6-phospho-gluconolactone was very limited, resulting in decreased formation of TPNH. Since the transformation of estrone to estradiol-17β was greatly limited in these subjects, this indicates that TPNH is the hydrogen donor for the reaction catalysed by estradiol-17β dehydrogenase. On the other hand, the transformation of estradiol-17β to estrone that required a hydrogen acceptor was normal in these subjects. The fact that in subjects with red cell glucose-6-phosphate dehydrogenase deficiency some estrone was transformed probably means that the deficiency was not complete. At the same time, if glucose-6-phosphate dehydrogenase is not completely absent, one would expect that enough TPNH would be generated to reduce the minute amounts of estrone that are being offered as substrate in the present experiments unless more active and more abundant TPNH-oxidizing systems are also present in the red cells. Such a possibility is to be considered, although it remains to be proved. The two dehydrogenases of the hexose monophosphate shunt are TPN-specific. The two dehydrogenases present in the Embden-Meyerhof-Parnas glycolytic pathway are DPN-dependent. In addition to the glucose-6-phosphate dehydrogenase system, one of the three other hydrogen transfers of the glycolysis could be coupled with the hydrogen transfer by the estradiol-17β dehydrogenase. The addition of DPN to hemolyzed red cells did not produce any significant transformation of estradiol-17β. Therefore, only the 6-phospho-gluconic dehydrogenase could possibly be coupled to the estradiol-17β dehydrogenase system; this, however, has not been determined.

The occurrence of pyridine nucleotide transhydrogenase activity in animal tissue mitochondria has been reported (25). An enzyme system which is specifically stimulated by estrogens added *in vitro* has been demonstrated in human placental preparations (26), and it was found that this system catalyzed a pyridine nucleotide transhydrogenation (27, 28). More recently, an estradiol-17β dehydrogenase has been separated from the estrogen-sensitive transhydrogenase (29). Whether the human red cell enzyme is identical with the estradiol-17β dehydrogenase found in human placenta has not been determined. Since it is impossible to remove all the blood contained in placental tissue, placental estradiol-17β dehydrogenase activity could be partially of red cell origin. The behavior of the placental system described by Ryan and Engel (30), however, would seem to argue against erythrocyte origin for the placental dehydrogenase.

The role and the importance of the estradiol-17β-dehydrogenase in human red cells is not known. The fact that this enzyme system is in large part coupled with the glucose-6-phosphate dehydrogenase might suggest that it plays a role in the glycolysis of the red cell. The slow rate of interconversion of estrone and estradiol-17β, however, would appear to limit the importance of this system. It should be added that in the presence of plasma, the extent of the interconversion is smaller. This is due probably to the fact that a large fraction of the steroid is bound to plasma protein and therefore unavailable to red cells (31–34). Since a similar situation would be found in the general circulation of man, this would also reduce the importance of the system *in vivo*.

Estradiol-17β is known to be a more biologically active estrogen than estrone. Bischoff and his collaborators (6) studied only the transformation of estrone to estradiol, and have considered that red cells contained an "activating" enzyme as far as biological activity is concerned. The reac-

tion is reversible, however, and the equilibrium depended upon glucose concentrations. It is not possible at the present time to tell where the equilibrium would be situated in *in vivo* conditions, nor can we state the *in vivo* importance of the enzyme system in metabolizing estrogens of endogenous origin.

SUMMARY

The presence of an estradiol-17β dehydrogenase in human red cells is confirmed. The enzyme is TPN-dependent. It is coupled with the glucose-6-phosphate dehydrogenase for the transformation of estrone to estradiol-17β. The thermolability and the pH optimum of the enzyme in intact cells has been determined. The red cells of subjects with glucose-6-phosphate deficiency have a marked but not complete decrease of the transformation, indicating that the interconversion of estrone and estradiol -17β is related to the activity of the hexose monophosphate shunt.

The physiological importance of the red cell estradiol-17β dehydrogenase remains to be determined.

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