BBA 65782

Δ^5-3-KETOSTEROID ISOMERASE ACTIVITIES IN GUINEA-PIG ADRENAL GLANDS: THE ROLE OF COPPER IONS

WOLFGANG EWALD* AND I. L. CHAIKOFF**

Department of Physiology, University of California, Berkeley, Calif. (U.S.A.) and Second Clinique of Internal Medicine, University of Frankfurt/Main, Frankfurt/Main, (Germany)

(Received April 1st, 1968)

SUMMARY

(1) Androst-5-ene-3,17-dione isomerase, pregn-5-ene-3,20-dione isomerase and 17α-hydroxypregn-5-ene-3,20-dione isomerase activities were found in the initial extract of guinea-pig adrenal glands and in its particulate cell fraction. The soluble cell fraction showed no activity and the particulate fraction contained not more than one third of the activity present in the initial extract. Combining the soluble with the particulate fraction augmented the isomerization rate considerably.

(2) Addition of copper ions (Cu^{2+}, Cu^{+}) to the soluble fraction activated the three isomerases in this cell fraction. The effect appears to specifically require copper ions.

(3) Both augmentation of the isomerization rate obtained with the combined soluble and particulate fractions and activation of the isomerizing capacity in the soluble fraction require a non-dialyzable factor besides copper ions.

(4) It appears that the two observations reflect the action of the same factor and that the catalytically active, non-dialyzable, thermolabile, and copper-sensitive factor in the soluble fraction is an enzyme protein lacking copper ions at the proper concentration for its activity.

(5) It is further suggested that the particulate fraction contains structurally bound ionic copper, thus providing ionic copper for the activity of the enzyme protein in the soluble fraction, when soluble and particulate fractions are combined.

(6) Finally, it appears that two isomerizing systems exist side by side, one associated with the cell particles, the other detected only in presence of the soluble fraction and copper ions.

INTRODUCTION

The existence of four Δ^5-3-ketosteroid isomerases in bovine adrenal gland was recently demonstrated in this and other laboratories[1-4]. The present report deals

* Present address: II. Medizinische Universitätsklinik, Frankfurt/Main, Germany.
** Deceased January 25, 1966.

with the androstenedione*, pregnenedione and 17-hydroxypregnenedione isomerase activities in the adrenal gland of another species, the guinea pig. While all three isomerases were detected in the particulate fraction (nuclei, mitochondria, microsomes) of the gland, none was found in the soluble fraction (the so-called cell sap). However, this fraction contained an isomerase enzyme system which could be activated by addition of copper ions. It appears from our experiments that two isomerase systems exist side by side, one located in the particulate cell fraction, the other detected only when the soluble and the particulate fraction are combined or when copper ions are added to the soluble fraction.

MATERIAL AND METHODS

Animals and their treatment

Male guinea pigs (500–650 g) that had been maintained on a nutritionally adequate diet (Purina Chow) supplemented with lettuce once a week, were killed by a blow on the head and bled from the neck. Both adrenal glands were rapidly excised, freed of fat, and weighed.

Adrenal preparations

The two whole adrenal glands (150–350 mg) excised from each animal were homogenized in 30 vol. (v/w) of a 0.3 M phosphate buffer (pH 7.0) and the homogenate was spun at 250 $\times$ g for 10 min at 3°. After removal of a surface lipid layer, the supernatant liquid (which is referred to as "initial extract"), was decanted and assayed for its isomerase activities and protein content (4.1 $\pm$ 0.5 mg/ml). After about 30 min the extract was centrifuged for 60 min at 80 000 $\times$ g, after which another fine lipid layer was removed from the surface of the supernatant fluid. The latter was decanted and its volume measured. It is designated here "soluble fraction" (protein content 1.9 $\pm$ 0.2 mg/ml). The pellet containing nuclei, mitochondria and microsomes was suspended, with the aid of a homogenizer, in a volume of 0.1 M phosphate buffer (pH 7.0), equal to that of the initial extract centrifuged, and this suspension is referred to as "pellet suspension" (protein content 2.1 $\pm$ 0.3 mg/ml).

Substrates

Androstenedione, pregnenedione and 17-hydroxypregnenedione were prepared according to the procedure of DJERASSI, EUGH AND BOWERS[5], and they were purified as described earlier[6–8].

Enzyme assays

Androstenedione isomerase, pregnenedione isomerase and 17-hydroxypregnenedione isomerase activities were determined as described previously[6,7,3] by recording the increase in absorbance at 249 mμ.

---

* Trivial names: androstenedione, androst-5-ene-3,17-dione; 17-hydroxypregnenedione, 17α-hydroxypregn-5-ene-3,20-dione; pregnenedione, pregn-5-ene-3,20-dione; androstenedione isomerase, androst-5-ene-3,17-dione $\Delta^{5(6)}$–$\Delta^{4(5)}$isomerase (EC group 5.3.3.); 17-hydroxypregnenedione isomerase, 17α-hydroxypregn-5-ene-3,20-dione $\Delta^{5(6)}$–$\Delta^{4(5)}$ isomerase (EC group 5.3.3); pregnenedione isomerase, pregn-5-ene-3,20-dione $\Delta^{5(6)}$–$\Delta^{4(5)}$isomerase (EC group 5.3.3.).

Assays of isomerase activities of combined pellet suspension and soluble fraction

Procedures were the same as described[6,7,3]. Three reaction mixtures, each with a final volume of 3.0 ml, were prepared as follows: (a) 2.85 ml of buffer *plus* 0.05 ml of pellet suspension plus 0.1 ml of substrate solution; (b) 2.80 ml of buffer *plus* 0.05 ml pellet suspension *plus* 0.05 ml of soluble fraction *plus* 0.1 ml of substrate solution; (c) 2.65 ml of buffer *plus* 0.05 ml of pellet suspension *plus* 0.20 ml of soluble fraction *plus* 0.1 ml of substrate solution. Three blanks containing the enzyme preparation(s) but no substrate, and another blank containing substrate but no enzyme served as controls. The isomerization rates in reaction mixtures (b) and (c) which exceeded the isomerization rate observed with the pellet alone (reaction mixture (a)), are referred to below as the "augmented isomerization rates" observed with the combined fractions.

Assay of activated isomerizing capacity in the soluble fraction by $Cu(NO_3)_2$

Except for one modification, assay procedure and composition of the reaction mixture were the same as those described[6,7,3] for the assays of androstenedione isomerase, pregnenedione isomerase and 17-hydroxypregnenedione isomerase activities; the volume of the triethanolamine–HCl buffer (0.1 M, pH 7.0) was 0.1 ml less than that indicated in the assay procedures: and 0.1 ml of a 15 mM solution of $Cu(NO_3)_2$ in 0.1 M triethanolamine–HCl buffer (pH 7.0) was added to the buffer prior to the additions of enzyme and substrate. In the presence of $Cu(NO_3)_2$ some proteins were precipitated, resulting in a slight increase of the enzyme blank. The substrate blank was considerably increased in the presence of $Cu(NO_3)_2$.

Protein determinations

The protein contents of the adrenal preparations were determined by the method of LOWRY *et al.*[9].

RESULTS

Isomerase activities of the initial extract, its soluble and particulate fractions and the combination of the two fractions in equal amounts

Androstenedione, pregnenedione and 17-hydroxypregnenedione isomerase were detected in the initial extract obtained by spinning the guinea-pig adrenal homogenate at 250 × *g* for 10 min (Table I). The activities were, however, not stable. After a rapid initial drop (15–30% during the first 15 min) there was a slight decrease until the activities became stable after about 20 h. The activities found in the initial extract after the first rapid drop, *i.e.* about 30 min after centrifugation of the adrenal homogenate, were used as a reference (control) value for all further experiments.

When the initial extract was dialyzed for 18 h at 3° against 100 vol. of 0.1 M phosphate buffer (pH 7.0) with stirring and several changes of the external fluid, 31% (23–44% in 13 animals) of the initial extract's activity was recovered, whereas 68% (41–75%) was found in the extract left standing at 3° for the same period.

Table I shows the three isomerase activities detected in the initial extract and in its soluble and particulate fractions. None of the three enzyme activities were detected in the soluble fraction. The pellet suspension contained only 21–67% of the isomerase activities present in the initial extract. But when equal amounts of

TABLE I

ANDROSTENEDIONE, PREGNENEDIONE AND 17-HYDROXYPREGNENEDIONE ISOMERASE ACTIVITIES IN CENTRIFUGAL FRACTIONS OF GUINEA-PIG ADRENAL GLANDS AND IN RECOMBINED FRACTIONS OF THE GLAND

The initial extract was obtained by low-speed centrifugation from the homogenate of the two adrenal glands of a single guinea pig, and it was separated into its soluble and particulate fractions as described in the experimental section. Isomerase activities were assayed in the initial extract, in its soluble and particulate fractions and in reaction mixtures containing equal amounts of these two fractions. The activities of androstenedione, pregnenedione and 17-hydroxypregnenedione isomerases detected in the initial extract 30 min after its preparation was attributed a value of 100%. 50 μl of each adrenal gland preparation were used to assay androstenedione isomerase activity, and 100 μl to assay pregnenedione and 17-hydroxypregnenedione isomerase activities. In reaction mixtures containing both soluble fraction and pellet suspension, the volume of each fraction was 50 μl for the assay of androstenedione isomerase activity and 100 μl for that of pregnenedione and 17-hydroxypregnenedione isomerase activities. The number of animals from which the values were obtained is indicated in parentheses.

Preparation	*Androstenedione isomerase activity % (23)*	*Pregnenedione isomerase activity, % (12)*	*17-Hydroxypregnenedione isomerase activity, % (8)*
Initial extract	100	100	100
Pellet suspension	36 (24–67)	39 (21–60)	32 (23–43)
Soluble fraction	0	0	0
Pellet suspension *plus* soluble fraction in equal concentrations	69 (54–91)	56 (33–75)	44 (29–54)

the soluble fraction and the pellet suspension were added to the reaction mixture, the isomerization rates of androstenedione, pregnenedione and 17-hydroxypregnenedione were augmented. The isomerization rate of androstenedione observed with the combined fractions was about twice, while the isomerization rates of 17-hydroxypregnenedione and pregnenedione were about 1.3–1.5 times that observed with the

TABLE II

EFFECT OF TEMPERATURE ON THE PELLET ACTIVITY AND ON THE AUGMENTED ISOMERIZATION RATES

Two 3-ml aliquots of the pellet suspension and two 3-ml aliquots of the soluble fraction were stored for 20 h, one aliquot of each preparation at 3°, the other at 25°. The androstenedione isomerase activities of the pellet suspension and the augmented isomerization rates of androstenedione were determined at $t = 0$ and after 20 h of storage at either 3° or 25°. The values are expressed as nmoles of androstenedione isomerized per min per ml of pellet suspension.

Fractions and volumes	*Control $t = 0$; 3°*	*After standing for 20 h at*	
		3°	*25°*
Pellet suspension alone	71	41	38
Pellet suspension (50 μl) *plus* 50 μl of soluble fraction	101	68	58
Pellet suspension (50 μl) *plus* 200 μl of soluble fraction	140	110	69

pellet alone (Table I). The augmented rates observed with the combined fractions varied among the animals and did not appear to depend upon the pellet's activity.

Effect of temperature on the androstenedione isomerase activities of the pellet suspension and of the combined fractions

Two aliquots of the pellet suspension and two of the soluble fraction were kept for 20 h, one of each at 3° and the other at 25°. Their isomerase activities were determined before and after this period (Table II). The activity of the pellet suspension decreased in the 20 h to about the same extent, regardless of whether it was kept at 3° or 25° (compare columns 3 and 4 with column 2). But the augmented isomerization rates observed with the combined fractions dropped to about one third of the original value when the soluble fraction was kept at 25°, (column 4), while the augmented rates were unchanged when the soluble fraction was kept at 3° (column 3).

These findings show that a factor in the soluble fraction necessary to obtain the augmented isomerization rates is sensitive to an increase in temperature from 3° to 25°, whereas the activity associated with the cell particles is not. The drop in isomerization rates observed with the combined fractions kept at 3° can be entirely accounted for by the drop in the pellet's activity (compare columns 2 and 3).

Activation of capacity to isomerize Δ^5-3-ketosteroids in the soluble fraction by copper nitrate

The observation that the androstenedione isomerase activity in bovine adrenal

TABLE III

EFFECT OF $Cu(NO_3)_2$ ON THE ANDROSTENEDIONE ISOMERASE ACTIVITIES IN ADRENAL PREPARATIONS

Androstenedione isomerase activity of the initial extract and of its soluble and particulate fractions were determined in 0.1 M triethanolamine–HCl buffer (pH 7.0) with and without addition of $5 \cdot 10^{-4}$ M (final concentration) $Cu(NO_3)_2$. The copper salt was dissolved in triethanolamine–HCl buffer and added to the buffer solution before addition of enzyme and substrate. Separate substrate and enzyme blanks were determined for reaction mixtures containing $Cu(NO_3)_2$ and those containing no salt. All assays were carried out with 50 µl of the pertinent adrenal preparation. The activity of the initial extract 30 min after its preparation without addition of $Cu(NO_3)_2$ to the reaction mixture was considered 100%. The values were obtained from 4 single guinea pigs.

Preparation	*Activity without addition of $Cu(NO_3)_2$ (%)*	*Activity with addition of $Cu(NO_3)_2$ (%)*
Initial extract	100	98 (85–112)
Soluble fraction	0	84 (65–103)
Pellet suspension	36 (27–56)	33 (26–40)
Pellet suspension *plus* soluble fraction	70 (58–86)	94 (76–110)

preparations* was increased by addition of $Cu(NO_3)_2$ led us to investigate the effect of copper ions on Δ^5-3-ketosteroid isomerase activities in the guinea-pig adrenal preparations. Table III records the androstenedione isomerase activities of the initial extract, its particulate and soluble fractions, and of a combination of these fractions, with and without addition of $Cu(NO_3)_2$.

Addition of 1.5 μmoles of $Cu(NO_3)_2$ to the reaction mixture did not significantly alter the isomerization rates of androstenedione in the initial extract nor in the pellet suspension. The augmented isomerization rates observed with the combined fractions in absence of copper ions was further enhanced by the addition of $Cu(NO_3)_2$; however, the rate did not exceed that of the initial extract. From the inability to enhance the pellet activity by addition of $Cu(NO_3)_2$ it appears that there is, besides ionic copper, a second factor in the soluble fraction which is required for the augmentation of the isomerization rates.

Moreover, a very high isomerization rate was observed when the soluble fraction, inactive by itself, was assayed after addition of $Cu(NO_3)_2$: the isomerization rate of androstenedione amounted to 84% of that detected originally in the initial extract. From this observation it may be concluded that a factor exists in the soluble

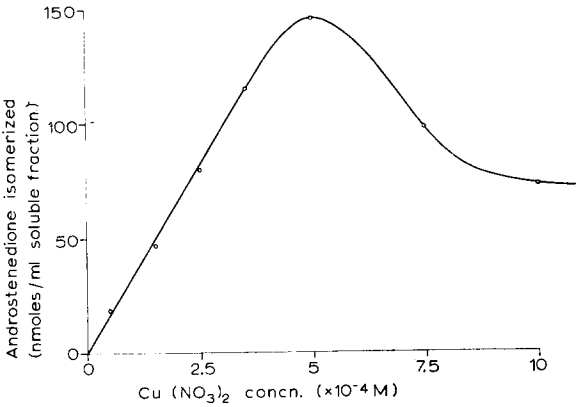

Fig. 1. Effect of addition of $Cu(NO_3)_2$ at various concentrations on the magnitude of activation of androstenedione isomerase in the soluble fraction. Copper solutions were prepared at concentrations suitable to yield the indicated final concentrations, and 0.1 ml of each was added to the buffer prior to addition of enzyme and substrate. Reactions are carried out as described in the experimental section.

fraction which, though unable to catalyze by itself the isomerization of androstenedione, acquires the capacity to catalyze the isomerization of androstenedione upon addition of $Cu(NO_3)_2$.

Fig. 1 shows the isomerization rates observed when the soluble fraction was assayed after addition of various amounts of $Cu(NO_3)_2$ to the reaction mixture. A linear increase of the isomerization rates was observed with concentrations of $Cu(NO_3)_2$ up to $4 \cdot 10^{-4}$ moles/l; at higher concentrations the isomerization rates declined. The

* W. EWALD AND I. L. CHAIKOFF, unpublished observations.

decrease probably results from denaturation of the factor in the soluble fraction requiring copper ions to elicite the isomerizing capacity.

Activation of isomerase activity in the soluble fraction was also observed with other Δ^5-3-ketosteroids. Table IV shows the isomerization rates of androstenedione, pregnenedione and 17-hydroxypregnenedione obtained after addition of $Cu(NO_3)_2$ to the soluble fraction and also the rates of the initial extract. Highest activation was observed with androstenedione, lowest with pregnenedione, whereas in the initial

TABLE IV

ACTIVATION OF ISOMERIZING CAPACITY BY $Cu(NO_3)_2$ IN THE SOLUBLE FRACTION

Androstenedione isomerase, pregnenedione isomerase and 17-hydroxypregnenedione isomerase activities were determined in the initial extract obtained from single guinea pigs without addition of $Cu(NO_3)_2$ and in the soluble fraction obtained from the initial extracts by centrifugation at 80 000 × *g* for 60 min without and with addition of $5 \cdot 10^{-4}$ M $Cu(NO_3)_2$ (final concentration). The number of animals is indicated in parentheses. The values are expressed as nmoles of steroid isomerized per min per ml of adrenal preparation.

Preparation	*Substrate*		
	Androstenedione (12)	*Pregnenedione (4)*	*17-Hyroxypregnenedione (4)*
Adrenal extract	196 (109–278)	62 (58–71)	42 (38–49)
Soluble fraction	0	0	0
Soluble fraction *plus* $Cu(NO_3)_2$	101 (42–190)	11 (4–20)	39 (26–53)

extract the lowest activity was found with 17-hydroxypregnenedione. No correlation was observed between the activity of the initial extract prepared from the adrenal glands of a particular guinea pig and the activated isomerization rate in the soluble fraction obtained from the glands of the same animal.

Requirement of copper ions for the augmented isomerization rates observed with the recombined fractions

Dialysis of the soluble fraction resulted in the loss of the augmented isomerization rates (Table V). To decide, whether the dialyzable component of the soluble fraction is ionic copper, two aliquots of the soluble fraction were dialyzed: one against the 0.1 M triethanolamine–HCl buffer (pH 7.0) and the other against the same buffer of pH 7.0 containing $5 \cdot 10^{-4}$ M Cu $(NO_3)_2$.A third aliquot served as control and was left standing at 3° for the period of dialysis (20 h). The pellet's activity was not affected by dialysis. Its activity dropped during the 20-h period to the same extent whether it had or it had not been dialyzed against the 0.1 M phosphate buffer (pH 7.0) (Table V, column 2).

When the aliquot of the soluble fraction which had been dialyzed against the 0.1 M triethanolamine–HCl buffer containing no $Cu(NO_3)_2$ was combined in the reaction mixture with the pellet suspension, the isomerization rates were not significantly augmented above that observed with the pellet suspension alone (Table V,

TABLE V

EFFECT OF DIALYSIS AGAINST TRIETHANOLAMINE–HCl BUFFER OF pH 7.0 WITH AND WITHOUT $Cu(NO_3)_2$ ON THE AUGMENTED ISOMERIZATION RATES OF THE COMBINED FRACTIONS AND ON THE ACTIVATION OF ISOMERIZING CAPACITY IN THE SOLUBLE FRACTION

A 5-ml aliquot of the pellet suspension was dialyzed with stirring against 100 vol. of 0.1 M phosphate buffer (pH 7.0) overnight with 3 changes of the external fluid. An aliquot of 3.0 ml of soluble fraction was dialyzed with strong stirring against about 500 ml of 0.1 M triethanolamine–HCl buffer (pH 7.0) overnight with 3 changes of the dialysis bath. Another aliquot of 5 ml was dialyzed in the same way against 0.1 M triethanolamine–HCl buffer of pH 7.0 containing $5 \cdot 10^{-4}$ M $Cu(NO_3)_2$. A portion of this aliquot was taken for the assays and the remainder was redialyzed with strong stirring against 0.1 M triethanolamine–HCl buffer (pH 7.0) for 48 h with 8–10 changes of the dialysis bath. The contents of the dialysis bag were used without previous centrifugation in the assays. 75 μl of pellet suspension *plus* the indicated volume of soluble fraction were used to determine the activity of the combined fractions. The isomerization rates of the soluble fraction were determined with 50 μl of the solution. $Cu(NO_3)_2$ was used in final concentration of $5 \cdot 10^{-4}$ moles/l. The values are expressed as nmoles of androstenedione isomerized per min per ml of adrenal preparation.

Treatment of soluble fraction	*Pellet activity and isomerization rates observed with the combined fractions*			*Isomerization rates observed in the soluble fraction*	
				Without addition of $Cu(NO_3)_2$	*With addition* $Cu(NO_3)_2$
μl of soluble fraction combined with 75 μl of pellet suspension	*0*	*75*	*200*		
Not dialyzed (t = 0)	61	90	124	0	115
Not dialyzed (after 20 h)	36	53	89	0	118
Dialyzed against 0.1 M buffer	34	41	43	0	125
Dialyzed against 0.1 M triethanolamine–HCl buffer containing $Cu(NO_3)_2$	34	52	123	49	48
Dialyzed against 0.1 M triethanolamine–HCl buffer containing $Cu(NO_3)_2$ and redialyzed against 0.1 M triethanolamine–HCl buffer without $Cu(NO_3)_2$ to remove Cu^{2+} ions	35	42	57	0	40

compare columns 3 and 4 with column 2). This finding shows that the presence of a dialyzable component in the soluble fraction is required to evoke the augmented isomerization rates. On the other hand, the capacity to isomerize androstenedione, was activated in this aliquot of the soluble fraction upon addition of copper ions to the reaction mixture (columns 5 and 6). This latter finding demonstrates that the factor of the soluble fraction requiring the copper ions for its isomerizing capacity is not dialyzable.

But when the aliquot of the soluble fraction that had been dialyzed against the 0.1 M triethanolamine–HCl buffer containing $Cu(NO_3)_2$ was combined with the pellet suspension highly augmented isomerization rates were observed (columns 2–4). From this finding it can be seen that the factor in the soluble fraction which, besides copper ions, is required for the augmentation of the isomerization rate, was not lost

during dialysis. Thus, both the augmentation of the isomerization rate and the activation of isomerizing capacity by $Cu(NO_3)_2$ require the presence of a non-dialyzable factor in the soluble fraction. Moreover, the aliquot of the soluble fraction that had been dialyzed against the copper containing buffer kept the capacity to isomerize androstenedione without further addition of $Cu(NO_3)_2$ to the reaction mixture (column 5). The magnitude of activation in the aliquot dialyzed against the triethanolamine–HCl buffer containing $Cu(NO_3)_2$ was only half that of the aliquot dialyzed against triethanolamine–HCl buffer without $Cu(NO_3)_2$ (column 6). This probably

TABLE VI

SUBSTITUTION OF VARIOUS IONS IN THE EFFECTS OBSERVED WITH $Cu(NO_3)_2$

A portion of the initial extract obtained from 2 or 3 guinea pigs was dialyzed at 3° against 50 vol. of 0.1 M phosphate buffer (pH 7.0) for 20 h with stirring and 2 changes of the external fluid. The remainder was centrifuged at 80 000 × *g* for 60 min. The pellet suspension and the soluble fraction were dialyzed separately at 3° overnight against the 50 vol. of 0.1 M triethanolamine–HCl buffer (pH 7.0) with stirring and 3 changes of the external fluid. The salt solutions were used without adjustment of the pH. The pH of the solutions was between 4.5 and 7.0 except for CuCl. This salt was dissolved in 0.05 M HCl and then added to the reaction mixture. The pH was decreased to 6.5. Assays of androstenedione isomerase activities were carried out as described in the experimental section. Other ions not listed in the table were also tested for their capacity to substitute for copper ions. Their concentrations were: NaCl: 100, 25, 5 mM; $CaCl_2$, $Zn(C_2H_3O_2)_2$: 25, 5, 1 mM; RbCl, $CdCl_2$, $StCl_2$: 10, 1 μM; $CoCl_2$, $NiCl_2$: 10, 2, 0.2 μM; $FeCl_2$, $FeCl_3$: 50, 10, 1 μM.

Preparation	*Salt added*	*Final salt concentration (M)*	*Activity of the initial extract (%)*	*Pellet activity* and isomerization rates observed with the combined fractions*			*Activation of isomerizing capacity** in the soluble fraction*
μl of soluble fraction combined with 50 μl of pellet suspension				*0*	*50*	*200*	
Original	None		100	48	73	129	
Dialyzed	None		31	36	48	61	
	$Cu(NO_3)_2$	$5 \cdot 10^{-4}$	105	63	173	305	49
		$1.5 \cdot 10^{-4}$		127	195	295	
		$5 \cdot 10^{-5}$		52	85	128	
	CuCl	$5 \cdot 10^{-4}$		82	80	150	52
		$1.5 \cdot 10^{-4}$		113	100	121	
		$5 \cdot 10^{-5}$		68	70	127	
	$AgNO_3$	$5 \cdot 10^{-5}$		22	45	104	5
		$1 \cdot 10^{-5}$		30	38	80	
		$1 \cdot 10^{-6}$		33	40	72	
	$MgCl_2$	$5 \cdot 10^{-4}$	30	24	27	48	0
		$1.5 \cdot 10^{-4}$		21	23	41	
	KCl	$5 \cdot 10^{-2}$	33	22	27	46	0
		$1 \cdot 10^{-2}$		20	27	41	

* Values expressed as nmoles of androstenedione isomerized per min per ml of pellet suspension.

** Values expressed as nmoles of androstenedione isomerized per min per ml of soluble fraction.

resulted from the precipitation of proteins by the dialysis against the $Cu(NO_3)_2$ containing buffer.

The aliquot of the soluble fraction dialyzed against 0.1 M triethanolamine–HCl buffer containing $5 \cdot 10^{-4}$ $Cu(NO_3)_2$ was then redialyzed with 8–10 changes for 2 days against 0.1 M triethanolamine–HCl buffer (pH 7.0) to remove the copper ions from the preparations. The results obtained with this aliquot were essentially the same as those obtained with the aliquot dialyzed against the buffer containing no copper ions.

These findings demonstrate that the dialyzable factor in the soluble fraction required to yield the augmented isomerization rates of androstenedione in combination with the pellet suspension is either ionic copper or can be replaced by $Cu(NO_3)_2$. Furthermore, it is shown that a non-dialyzable factor is involved in the augmentation of the isomerization rates as well as in the elicitation of isomerizing capacity in the soluble fraction by cuprous nitrate.

Specificity of Cu^{2+} ions

The specificity of copper ions in their effects on the androstenedione isomerase activity was examined by three tests: (a) restoration, by addition of various ions, of the isomerase activity lost by dialysis of the initial extract; (b) restoration, by addition of various ions, of the soluble fraction's ability to yield augmented isomerization rates in combination with the pellet suspension lost after dialysis of the soluble fraction; (c) activation, by addition of various ions, of the capacity to isomerize androstenedione in the soluble fraction.

The efficiencies of Cu^{2+}, Cu^{+}, Ag^{+}, Mg^{2+}, and K^{+} in these three tests at approximately physiological concentrations[11], are listed in Table VI. The effects of Na^{+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, St^{2+}, Rb^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} and Co^{2+} were also tested at approximately physiological concentration (see legend for Table VII).

Addition of $Cu(NO_3)_2$ in concentrations ranging from $5 \cdot 10^{-4}$ to $5.0 \cdot 10^{-5}$ M resulted in a magnification of the augmented isomerization rates (columns 5–7). A slight enhancement of the pellet's activity by $Cu(NO_3)_2$ was noted which was not detected with the non-dialyzed pellet suspension (Table V).

Addition of Cu^{+} ions activated the capacity to isomerize androstenedione in the soluble fraction to the same extent as did Cu^{2+} ions (column 8). Their effect on the augmented isomerization rates observed with the combined fractions was a little less than that of Cu^{2+} ions (columns 5–7).

Ag^{+} ions were slightly effective both in the augmentation of the isomerization rates and in the activation of isomerizing capacity in the soluble fraction. However, the efficacy did not exceed one tenth of that observed with the copper ions though it was consistently observed.

None of the other ions tested showed a measurable effect in the enhancement of the augmented isomerization rates or in the activation of isomerizing capacity or in the restoration of the activity of the dialyzed initial extract. Thus, the three effects observed with $Cu(NO_3)_2$ were obtained almost exclusively with copper ions. Cu^{2+} appeared to be somewhat more effective than Cu^{+}. The only other effective ion was Ag^{+} but its effect was only slight.

DISCUSSION

Several of our observations on the augmented isomerization rates and on the activated isomerizing capacity differed from those on the isomerase activity associated with the cell particles: (a) the pellet activity was unstable at 3°, while the augmented isomerization rates observed with the combined fractions were unchanged (Table II); (b) raising the temperature to 25° did not magnify the drop of the pellet activity observed at 3°, while two third of the augmented isomerization rates were lost after standing for 20 h at that temperature (Table II); (c) the pellet activity was the same after dialysis, whereas the augmentation of the isomerization rates was lost after dialysis of the soluble fraction (Table V); (d) addition of Ag^+ decreased the activity of the pellet suspension (Table VI), but addition of this ion augmented the isomerizing capacity in the soluble fraction (Table VI).

On the other hand, the similarities between the activated isomerizing capacity and the augmented isomerization rates observed with the combined fractions are obvious. In both cases a dialyzable factor in the soluble fraction was required (Tables V and VI). Only copper and silver ions activated the isomerizing capacity in the soluble fraction and only these ions yielded augmented isomerization rates after the soluble fraction had been dialyzed (Table VI). In both cases Cu^{2+} and Cu^+ ions were about equally effective, and the efficacy of Ag^+ ions was only small (Table VI).

Moreover, an additional factor in the soluble fraction is involved, both in the augmentation of the isomerization rates by the combined fractions (Tables III and V) and in the activation of isomerizing capacity by copper ions (Tables III and V). In each case, the factor was found to be non-dialyzable (Table V), and it required copper ions for its catalytic activity. Further, the augmented isomerization rates were found to be thermolabile (Table II), and the copper induction was sensitive to high concentrations of copper ions (Fig. 1).

Thus, it seems reasonable to suggest that both augmentation of the isomerization rate and activation of isomerizing capacity reflect the action of the same factor in the soluble fraction, and that the catalytically active, non-dialyzable, thermolabile and copper sensitive factor may be an enzyme protein lacking copper ions at the proper concentration for its activity.

If the isomerizing capacity activated by the copper ions and the augmented isomerization rates observed with the combined fractions were brought about by two distinct isomerizing systems, their rates would be expected to be additive. However, this was not the case: combination of soluble fraction, pellet suspension and copper ions in the same reaction mixture yielded the same rate as that of the initial extract (Table III), whereas mere numerical addition of the isomerization rate obtained by copper activation (line 2, column 3) *plus* that of the combined fractions (line 4, column 2) would amount to one and a half that of the initial extract (Table III). The inability to increase the isomerase activity in our assay by combination of pellet suspension, soluble fraction and copper ions over that of the initial extract thus supports our view that augmented isomerization rates and activation of isomerizing capacity by copper ions result from the action of the same factor in the soluble fraction which requires copper ions for its activity.

Since the augmented isomerization rates were lost after dialysis of the soluble fraction and were restored by addition of copper ions only (Table V and VI), it

appears that some copper ions are present in the soluble fraction although their concentration is not high enough to elicit an isomerizing capacity in the soluble fraction (Tables III and IV, Fig. 1).

In view of the specific requirement for copper ions to obtain both activated isomerizing capacity and augmented isomerization rates (Table VI) it is suggested that the pellet suspension may contain some ionic copper bound to its structural proteins. By combining the pellet suspension with the soluble fraction the concentration of ionic copper in the reaction mixture would be increased, resulting in some isomerizing capacity of the factor in the soluble fraction requiring, though lacking, copper ions at the proper concentration. However, the possibility cannot be ruled out that a component other than ionic copper is associated with the particles of the pellet suspension and acts in combination both with the non-dialyzable, thermolabile factor and the copper ions of the soluble fraction to yield the augmented isomerization rates.

ACKNOWLEDGEMENT

The work was supported by a U.S. Public Health grant (CA-00879-17).

REFERENCES

1 W. EWALD, H. WERBIN AND I. L. CHAIKOFF, *Biochim. Biophys. Acta*, 81 (1964) 199.
2 H. L. KRÜSKEMPER, E. FORCHIELLI AND H. J. RINGOLD, *Steroids*, 3 (1964) 295.
3 W. EWALD, H. WERBIN AND I. L. CHAIKOFF, *Biochim. Biophys. Acta*, 111 (1965) 306.
4 A. ALFSEN, E. E. BAULIEU AND M. J. CLAQUIN, *Biochem. Biophys. Res. Commun.*, 20 (1965) 251.
5 C. DJERASSI, R. R. EUGH AND A. BOWERS, *J. Org. Chem.*, 21 (1956) 1547.
6 W. EWALD, H. WERBIN AND I. L. CHAIKOFF, *Steroids*, 4 (1964) 759.
7 W. EWALD, H. WERBIN AND I. L. CHAIKOFF, *Steroids*, 3 (1964) 505.
8 W. EWALD, H. WERBIN AND I. L. CHAIKOFF, *Biochim. Biophys. Acta*, 130 (1966) 556.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
10 S. P. L. SØRENSEN, *Ergeb. Physiol. Biol. Chem. Exptl. Pharmakol.*, 12 (1912) 393.
11 W. J. GOFMAN, in C. A. TOBIAS AND J. H. LAWRENCE, *Advances in Biological and Medical Physics*, Vol. 8, Academic Press, New York, 1962, p. 1.