THE EFFECT OF CALCIUM IONS AND FREEZING ON THE *IN VITRO* SYNTHESIS OF PREGNENOLONE BY RAT ADRENAL PREPARATIONS

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(Received May 6th, 1961)

SUMMARY

In the presence of a TPNH-generating system the synthesis of pregnenolone from endogenous precursors by the large particles (pellet 2) of rat adrenals is stimulated by Ca^{2+}. Ba^{2+} and Sr^{2+} at higher concentrations can replace Ca^{2+}. The stimulation does not appear to be due to the activation of a proenzyme. At the pH optimum of 6.2, freezing pellet 2 decreases pregnenolone synthesis. However, at pH 7.5 the previously frozen pellet 2 is more active than the normal one. These results are discussed with respect to the Ca^{2+} and freezing stimulation of corticoid synthesis in adrenal whole homogenates.

In the presence of TPNH and at pH 6.2, Ca^{2+} stimulates the formation of pregnenolone from cholesterol but not from 20α-hydroxycholesterol, but at pH 7.5 the latter reaction is stimulated. Also, in whole homogenates at pH 7.5, Ca^{2+} stimulates the transformation of 20α-hydroxycholesterol to corticoids.

Evidence is presented that the stimulation by freezing of both pregnenolone and corticoid synthesis at pH 7.5 appears to be due to the stimulation of the conversion of cholesterol to 20α-hydroxycholesterol. Several observations also indicate that this step is rate limiting in the transformation of cholesterol to corticoids in adrenal homogenates.

INTRODUCTION

It has been shown in a previous study[1] that corticoid production by rat adrenal homogenates, in the presence of TPN and glucose 6-phosphate, is stimulated by freezing the homogenate and by the presence of Ca^{2+}. It has also been found[2] that corticoid production from endogenous precursors requires the presence of the large particles (pellet 2) and the supernatant. The stimulation resulting from freezing is a consequence of events taking place in pellet 2. This fraction is also the rate-limiting fraction and contains most of the cholesterol present in the two fractions needed for corticoid production. In addition, in the sequence of reactions leading to the synthesis of corticoids from endogenous precursor, the steps stimulated by both freezing and Ca^{2+} appear to be concerned with the transformation of cholesterol to pregnenolone[2]. Since this transformation occurs in the mitochondria[3], it was concluded that Ca^{2+} also

probably acted on the pellet-2 fraction although direct evidence for this could not be obtained at that time.

Recent work by HALKERSTON *et al.*[4,5] indicates that the conversion of cholesterol to pregnenolone in adrenal mitochondria proceeds readily if TPNH is provided. On the basis of this report the synthesis of pregnenolone in pellet-2 preparations has been investigated with the aim to define some of the characteristics of this system and to establish more precisely the location of the stimulatory effects of Ca^{2+} and of freezing.

EXPERIMENTAL

The rat adrenal tissue used in this study was routinely preincubated as previously described[1] and pellet-2 was isolated from 0.154 *M* KCl homogenates according to the procedures already reported[2]. The homogenates usually contained 80–90 mg wet wt. of tissue/ml. Before use, pellet 2 was resuspended in 0.154 *M* KCl to a final volume equal to the volume of the original homogenate. Results expressed on a per 100 mg tissue basis refer to the wet wt. of tissue in the unfractionated homogenate. In one determination, the pellet-2 suspension obtained from a whole homogenate containing 82 mg wet wt. of tissue/ml contained 2.1 mg of protein/ml as determined by the method of LOWRY *et al.*[6] using crystalline bovine serum albumin as a standard. In this paper pellet-2 preparations which have not been frozen are called normal preparations.

The determination of pregnenolone

The procedure used is a modification of that of MUNSON *et al.*[7]. A suitable aliquot of the incubation mixture (usually 1.5 ml of an incubation mixture of 2.0 ml) is transferred to a glass-stoppered test tube and 2.5 ml of spectrograde dichloromethane is added. The tube is shaken vigorously for 1 min and then centrifuged to clearly separate the two layers. A 2.0-ml aliquot of the dichloromethane layer is removed and transferred to another glass-stoppered test tube. The dichloromethane is evaporated in a warm water bath under a stream of dry nitrogen and to the residue 0.2 ml of glacial acetic acid, 0.5 ml of the furfural reagent[7], and 1.7 ml of 16 *N* H_2SO_4 are added, with shaking after each addition. The tubes are placed in a water bath at 67° for 15 min, cooled in an ice bath, and 3.0 ml of dichloromethane added. The tubes are now shaken vigorously for 1 min and again centrifuged. The color appears in the dichloromethane layer and is read against dichloromethane at 677 mμ.

The reduction in the amount of reagents in the proportions indicated leads to about a four-fold increase in sensitivity, so that after extraction with 3 ml of dichloromethane, 1.0 μg of pregnenolone will give an absorbancy of about 0.014 in a cell of 1 cm width in the Beckman DU spectrophotometer. This sensitivity can be further increased by extraction with smaller volumes of dichloromethane. The extraction by dichloromethane after color development is necessary because the presence of lipids in the dichloromethane extracts of incubation mixtures containing crude tissue preparations results in turbid solutions in the reagents used for the color development. The extraction with dichloromethane results in a shift of the absorbancy maximum from 660 mμ to 677 mμ with a slight loss in sensitivity (approx. 8%). Other solvents such as hexane and 2,2,4-trimethylpentane are also effective in removing the lipid turbidity but they offered no advantages over dichloromethane and had some disadvantages, such as higher blank values, somewhat lower sensitivity and retention of the chromogen in the aqueous phase.

MUNSON *et al.*[7] have explored the specificity of the furfural procedure quite extensively and from their results substances other than pregnenolone expected to be encountered under the incubation conditions employed would not interfere. The modified procedure has been tested with cholesterol, progesterone, deoxycorticosterone, hydrocortisone, and 20α-hydroxycholesterol. None of these substances give appreciable color (absorbancy < 0.008 at the 200-μg level). As reported by MUNSON *et al.*[7] for dehydroisoandrosterone, the concentration curve deviates from linearity. At absorbancy readings greater than 0.09, values must be read from a standard curve.

MATERIALS

Sodium glucose 6-phosphate, NaTPN, NaTPNH, and glucose 6-phosphate dehydrogenase and pregnenolone were obtained from the Sigma Chemical Company, The 20α-hydroxycholesterol was a gift from Dr. M. GUT AND K. SHIMIZU.

RESULTS

Some properties of the system

Pellet-2 preparations fortified with TPN, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase show an increased synthesis of pregnenolone from endogenous precursors in the presence of Ca^{2+} (Fig. 1). However, freezing the pellet-2 preparations results in a diminution of pregnenolone synthesis and there is a relatively smaller Ca^{2+} effect. The Ca^{2+} ion is more effective in stimulating pregnenolone synthesis than any other cation tested. The data of Table I shows that Ba^{2+} and Sr^{2+} at higher concentrations can replace Ca^{2+}, while Mg^{2+} gives only a slight stimulation. Other cations tested had no effect or were inhibitory. Mn^{2+}, Co^{2+} and Ni^{2+} had no effect up to 5 m*M*, 1.0 m*M*, and 1.5 m*M* final concentration, respectively. Zn^{2+} became inhibitory at 0.2 m*M*, Fe^{2+} at 2.0 m*M*, Fe^{3+} at 1.0 m*M*, Cu^{2+} at 0.005 m*M* and Cu^{+} at 0.05 m*M*, all final concentrations.

The stimulation by Ca^{2+} could be due to the activation of a proenzyme to an enzyme, such as the transformation of prothrombin to thrombin, or Ca^{2+} may be a co-factor as in apyrase. In the first case preincubation with Ca^{2+} should result in an

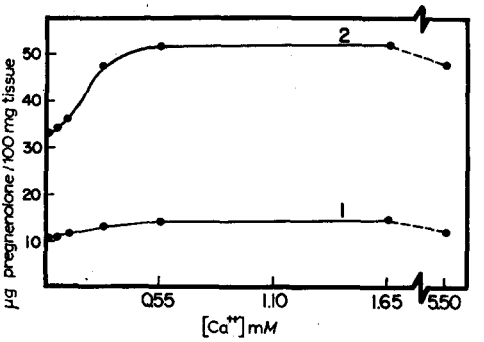

Fig. 1. The medium contained pellet 2, 90 μmoles phosphate buffer (pH 6.2), 1.6 mg NaTPN, 2.1 mg glucose 6-phosphate, 0.05 units glucose 6-phosphate dehydrogenase, and 0.154 *M* KCl and the indicated additions to a final volume of 2.0 ml. Incubation was for 2.0 h at 37° in air. Curve 1, the pellet-2 preparation had been previously frozen in a dry ice–ethanol mixture. Curve 2, normal pellet-2 preparation.

TABLE I

STIMULATION OF PREGNENOLONE SYNTHESIS BY VARIOUS CATIONS

The incubation medium contained 0.3 ml of pellet 2, 90 μmoles phosphite buffer (pH 6.2), 1.6 mg NaTPN, 2.1 mg glucose 6-phosphate, 0.05 units of glucose 6-phosphate dehydrogenase, the indicated cations, and 0.154 *M* KCl to a final volume of 2.0 ml. The values under the Ca^{2+} heading are control values in the absence or presence of Ca^{2+}. Incubation was for 1.0 h at 37° in air.

	Pregnenolone formed		
	Sr^{2+}	Ba^{2+}	Mg^{2+}
mM	*(μg/100 mg tissue)*		
0.2	23.2	20.8	25.3
0.5	27.5	24.1	23.7
1.5	41.4	29.2	27.0
5.0	41.1	36.9	27.5
15.0	40.0	46.8	
	Ca^{2+}	Ca^{2+}	Ca^{2+}
0	23.5	23.0	23.5
0.55	37.4	41.6	37.4

activation which will persist after the Ca^{2+} is removed with EDTA. In the second case the stimulation of the system will require the continuous presence of Ca^{2+}. It may be seen from the data of Table II that no evidence of stimulation during the preincubation with Ca^{2+} was obtained, in the sense of a persistence of the Ca^{2+} effect after the addition of EDTA. Since the Ca^{2+} stimulation may require the conditions needed for pregnenolone synthesis, *i.e.*, the presence of TPNH, the experiment described in Table III was run. It is apparent that since the pregnolone formed (*b*—*a*) is not greater than the pregnolone formed in Section C, that the addition of EDTA to pellet 2, which had been incubated with both Ca^{2+} and the TPNH-generating system, results in a reduction of the rate of pregnenolone synthesis to values obtained in the

TABLE II

EFFECT OF PREINCUBATION OF PELLET 2 IN THE PRESENCE OF Ca^{2+} ON PREGNENOLONE SYNTHESIS

Preincubation: The medium contained 0.3 ml of pellet 2, 90 μmoles of Na phosphite buffer (pH 6.2), 1.1 μmole of Ca^{2+} when present, and 0.154 *M* KCl to a final volume of 2.0 ml. Incubation was for the indicated times at 37° in air. Final incubation: To the beakers which had been preincubated were added 7.5 μmoles phosphite buffer (pH 6.2), 1.6 mg of NaTPN, 2.1 mg of glucose 6-phosphate, 0.05 units of glucose 6-phosphate dehydrogenase, and where indicated 1.1 μmole of Ca^{2+} and 2.0 μmoles of EDTA. 0.154 *M* KCl was added to a final volume of 2.2 ml. Incubation was for 1.0 h at 37° in air.

Preincubation		*Final incubation*			
Time	Ca^{2+}	*No addition*	Ca^{2+}	*EDTA*	Ca^{2+} + *EDTA*
(min)		*(μg pregnenolone/100 mg tissue)*			
0	—	17.5	32.6	18.0	17.5
10	—	18.0		18.0	
10	+	31.9		18.0	
30	—	17.8		19.1	
30	+	32.4		21.5	
60	—	18.3		18.0	
60	+	28.2		23.3	

case where Ca^{2+} and EDTA had been present from the start of the incubation. It would appear that the continuous presence of Ca^{2+} is needed for the system to become and remain stimulated.

Fig. 2A shows the variation in pregnenolone synthesis from endogenous substrates with pH with both phosphite and bicarbonate buffers under a variety of conditions. A pH of about 6.2 was optimal for pregnenolone synthesis in the absence and presence of Ca^{2+} and phosphite buffer at pH 6.2 was used routinely. It is to be noted that although freezing pellet 2 has a deleterious effect at the lower pH values, at pH values greater than 7.2 it is more active than the non-frozen preparation.

Since the TPNH requirement for this reaction, demonstrated by HALKERSTON *et al.*[4], is met by the generation of TPNH from glucose 6-phosphate and TPN in the presence of glucose 6-phosphate dehydrogenase, it was of consequence to determine whether the stimulation by Ca^{2+} operated through this reaction. It may be seen from Fig. 2B that the Ca^{2+} stimulation also takes place when TPNH is directly provided. The pH optimum has shifted from about 6.2 to about 5.8. This lower pH probably reflects more accurately the pH optimum of the series of reactions involved in the synthesis of pregnenolone in this preparation. It should be noted that here also the frozen pellet-2 preparation is more active than the non-frozen one at higher pH values.

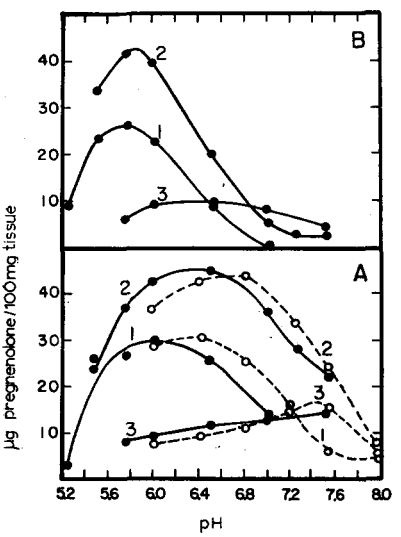

Fig. 2. The effect of pH on pregnenolone synthesis in pellet-2 preparations. Curve 1, pellet 2. Curve 2, pellet 2 plus Ca^{2+} at 0.55 m*M* final concentration. Curve 3, the pellet-2 preparation had been previously frozen in a dry ice–ethanol mixture. A, the medium contained the pellet-2 preparation, 1.6 mg of NaTPN, 2.1 mg. Na glucose 6-phosphate, 0.05 units of glucose 6-phosphate dehydrogenase, buffer and 0.154 *M* KCl to a final volume of 2.0 ml. Incubation was for 2.0 h at 37°. ●—●, 90 µmoles of acetate–phosphite buffer at the indicated pH values. Incubation was in air. ○—○, 0.154 *M* $NaHCO_3$ at volumes calculated to give the indicated pH values under an atmosphere containing 5% CO_2 and at 37° (see ref. 8). Incubation was in O_2–CO_2 (95:5). B, the medium contained the pellet-2 preparation, 90 µmoles of acetate–phosphite buffer at the indicated pH values, 0.8 mg of the NaTPNH, and 0.154 *M* KCl to a final volume of 2.0 ml. Incubation was for 2.0 h at 37° in air.

Fig. 3A describes the time course of the reaction in the presence and absence of Ca^{2+}. Little effect of the Ca^{2+} is seen in less than a 20-min incubation. It appears that in the absence of Ca^{2+}, pregnenolone synthesis may start at a rate comparable to that obtained in the presence of Ca^{2+}, but that this higher rate is maintained for only a short time. The effect of pellet-2 concentration is seen in Fig. 3B. Unlike the curves obtained with whole adrenal homogenates in the synthesis of corticoids from endogenous precursors[1], these curves do not exhibit increasing slopes. This suggests that the increasing slopes seen in the whole-homogenate system is due to the existence of a multiplicity of rate-limiting steps as proposed by WU AND RACHER[9] in their investigation of the rate of glycolysis in tumor-cell extracts and not to the presence of endogenous substrate[10].

TABLE III

NECESSITY FOR THE CONTINUOUS PRESENCE OF Ca^{2+} FOR STIMULATION OF PREGNENOLONE SYNTHESIS

The medium contained 0.3 ml of pellet 2, 90 μmoles of phosphite buffer (pH 6.2), 1.6 mg of NaTPN, 2.1 mg of glucose 6-phosphate, 0.2 units of glucose 6-phosphate dehydrogenase, and, when present, 1.1 μmole of Ca^{2+} and 2.0 μmoles of EDTA. 0.154 *M* KCl was added to a final volume of 2.0 ml. Appropriate controls were run with no TPN or glucose 6-phosphate present. Incubation was at 37° in air. In Section A, the beakers were incubated for the indicated times in the presence of Ca^{2+}. In Section B, EDTA was added to beakers which had already been incubated in the presence of Ca^{2+} as in Section A and the incubation continued for the additional times indicated. Thus, pregnenolone formed (*b–a*) represents the pregnenolone synthesis obtained after addition of EDTA to beakers which had been incubated with Ca^{2+} in the absence of EDTA for various times. In Section C, the Ca^{2+} and EDTA were both present from the start of the incubation.

A			*B*				*C*		
Incubation time (min)	*Addition*	*Pregnenolone formed (a) (μg/100 mg tissue)*	*Additional incubation time (min)*	*Additions*	*Pregnenolone formed (b)*	*Pregnenolone formed (b–a)*	*Incubation time (min)*	*Additions*	*Pregnenolone formed (μg/100 mg tissue)*
					(μg/100 mg tissue)				
15	Ca^{2+}	11.3	45	Ca^{2+} + EDTA	28.3	17.0	45	Ca^{2+} + EDTA	22.0
30	Ca^{2+}	26.7	30	Ca^{2+} + EDTA	37.7	11.0	30	Ca^{2+} + EDTA	12.6
50	Ca^{2+}	41.5	10	Ca^{2+} + EDTA	47.5	6.0	10	Ca^{2+} + EDTA	6.0
60	Ca^{2+}	44.0							
60		27.3							

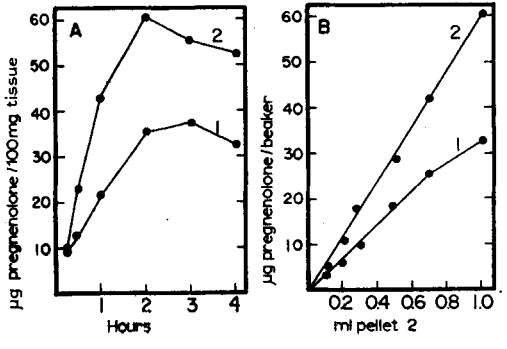

Fig. 3. A, time course of pregnenolone synthesis. The medium contained 0.3 ml of pellet 2, 90 μmoles of phosphite buffer (pH 6.2), 2.1 mg of glucose 6-phosphate, 1.6 mg of NaTPN, 0.05 units of glucose 6-phosphate dehydrogenase, and 0.154 *M* KCl to a final volume of 2.0 ml. Incubation was at 37° in air for the indicated times. B, the effect of tissue concentration on pregnenolone synthesis. The medium additions were as described in A. 1 ml of the pellet-2 preparation contained tissue corresponding to 90 mg wet wt. of the original whole homogenate. Incubation was for 2.0 h at 37° in air. Curve 1, no Ca^{2+} present. Curve 2, 0.55 m*M* Ca^{2+} present.

Various other properties of the system were also investigated. It is relatively insensitive to hypotonic conditions. The replacement of 0.5 ml of the 0.154 *M* KCl by water had little effect on pregnenolone synthesis in the presence or absence of Ca^{2+}, while the replacement of 0.87 ml of the KCl by water resulted in a 17% inhibition in the absence of Ca^{2+} and a 13% inhibition in the presence of Ca^{2+}. Hypertonic sucrose (0.87 *M* final concentration) inhibited synthesis 42% and 20% in the absence and presence of Ca^{2+}, respectively, and KCl at 0.4 *M* final concentration inhibited 63% in the presence or absence of Ca^{2+}. The inhibitory effect of high KCl concentration has also been seen in the synthesis of corticoid by whole homogenates[1].

Ascorbic acid over the concentration range 1.0 m*M* to 40 m*M* and cysteine and glutathione over the concentration range 0.75 m*M* to 30 m*M* stimulate pregnenolone synthesis only slightly (about 10%). Antimycin at 0.25 and 0.5 μg/ml, aminopterin over the concentration range 0.05 m*M* to 1.0 m*M*, and Amytal over the concentration range 0.2 m*M* to 2.0 m*M* had no effect on pregnenolone synthesis in the presence or absence of Ca^{2+}. *p*-Chloromercuriphenyl sulfonate was completely inhibitory at 0.1 m*M* final concentration.

In spite of the specificity displayed by the furfural procedure, additional evidence was deemed necessary to indicate that it was indeed pregnenolone that was being measured under incubation conditions. This was done by demonstrating that the furfural chromogen produced by the pellet-2 system is readily converted to corticoids in whole adrenal homogenates and by the demonstration that the increase in furfural chromogen resulting from the addition of TPNH and of TPNH plus Ca^{2+} to the incubation medium was reflected in increases in pregnenolone as detected chromatographically. It may be seen from the data of Table IV that when dichloromethane

TABLE IV

THE CONVERSION OF FURFURAL CHROMOGEN TO CORTICOIDS BY A WHOLE ADRENAL HOMOGENATE

Pellet-2 system: The medium contained 0.3 ml of pellet 2, 90 μmoles of phosphite buffer (pH 6.2), and when present, 1.6 mg NaTPN, 2.1 mg glucose 6-phosphate, 0.05 units of glucose 6-phosphate dehydrogenase and 1.1 μmole Ca^{2+}. 0.154 *M* KCl was added to a final volume of 2.0 ml. Incubation was for 1.0 h at 37° in air. Pregnenolone analysis by the furfural procedure and corticoid analysis by the blue tetrazolium method[11] were carried out on two identical sets of vessels and 2.0 ml of the dichloromethane extract of a third set was evaporated in the beakers in which the whole homogenate system incubation was then carried out. Whole homogenate system: In addition to the above mentioned evaporated dichloromethane extract, the medium contained 0.3 ml of the whole homogenate from which pellet 2 had been prepared, 40 μmoles of $NaHCO_3$, 1.6 mg NaTPN, 2.1 mg glucose 6-phosphate, and 0.154 *M* KCl to a final volume of 2.0 ml. Incubation was for 1.0 h at 37° under O_2–CO_2 (95:5). The corticoid-formed values in this system have been corrected for the small amount of blue tetrazolium-positive material present in the dichloromethane extracts from the pellet-2 system.

Pellet-2 system		*Whole homogenate system*	
Additions	*Furfural chromogen as μg pregnenolone/ 2 ml dichloromethane extract*	*Corticoid formed*	*Increased corticoid due to furfural chromogen addition*
		(μg/beaker)	
		8.46	
	0.41	9.07	0.6
Ca^{2+}	1.04	9.13	0.7
Glucose 6-phosphate dehydrogenase + glucose 6-phosphate + TPN	4.05	10.8	2.3
Glucose 6-phosphate dehydrogenase + glucose 6-phosphate + TPN + Ca^{2+}	7.34	13.4	4.9

TABLE V

CHROMATOGRAPHIC EVIDENCE THAT THE FURFURAL CHROMOGEN (FC) MEASURES PREGNENOLONE IN DICHLOROMETHANE EXTRACTS OF VARIOUS REACTION MIXTURES

Incubation condition I: each beaker contained 0.3 ml of pellet 2, 90 μmoles of phosphite buffer (pH 6.2), and 0.154 *M* KCl to a final volume of 2.0 ml. Incubation condition II: additions were the same as for condition I plus 1.6 mg of NaTPN, 2.1 mg of Na glucose 6-phosphate, and 0.10 unit of glucose 6-phosphate dehydrogenase. Incubation condition III: additions were the same as for condition II plus 1.1 μmole of Ca^{2+}. All incubations were for 1.0 h at 37° in air. The contents of 12 beakers for each group were pooled and extracted 3 times with dichloromethane. After removal of aliquots for analysis, the extract was chromatographed on paper using a ligroin–propylene glycol system[12] (system A). The pregnenolone areas were detected by an antimony trichloride reagent[13]. After elution of the areas corresponding to pregnenolone and removal of aliquots for analysis, the eluate was rechromatographed using the gas-chromatography procedure described by VANDEN HEUVEL *et al.*[14] (system B). The retention time of the experimental samples were identical with that of authentic pregnenolone. Concentrations were calculated on the basis of the areas under the curves, using pregnenolone as a standard.

Incubation condition	*FC found* (μg)	*Chromatography system*			
		A		*B*	
		FC put on paper (μg)	*FC found* (μg)	*FC put on column* (μg)	*Found*
I	3.1	1.7	1.7	1.2	0
II	33.8	26.7	17.1	11.7	13.3
III	74.3	59.6	41.6	28.3	27.5

extracts of the pellet-2 system containing varying amounts of furfural chromogen (depending on the incubation conditions of the pellet-2 system) are transferred to an adrenal whole homogenate system known to be able to transform pregnenolone to corticoids[2], the amount of corticoid formed is seen to depend on the amount of furfural chromogen added. In addition, the per cent transformation of the furfural chromogen is in the correct range to assume that all the furfural chromogen is pregnenolone[2]. The data of this table also confirm the results of HALKERSTON *et al.*[4] as to the necessity of the components of a system supplying TPNH for pregnenolone formation. Table V shows that by two chromatographic systems, the increases in furfural chromogen resulting from the addition of TPNH and of TPNH plus Ca^{2+} are reflected in increases in pregnenolone. In the paper-chromatographic system used 65–70% of the furfural chromogen is recovered in the pregnenolone zone. However, in control experiments using pregnenolone and the above paper-chromatographic system recoveries of 62–65% were obtained. This compares quite favorably with the experimental results and indicates that the furfural chromogen measured only pregnenolone. Dehydroisoandrosterone, which also reacts with the furfural reagent, could be present in the incubation system used. However, none of this substance could be detected chromatographically.

The location of the stimulation by Ca^{2+} and by freezing

The biochemical reactions involved in the conversion of cholesterol to pregnenolone are not well known. SOLOMON *et al.*[15] have isolated 20α-hydroxy-[4-^{14}C]cholesterol after incubation of [4-^{14}C]cholesterol with adrenal homogenate and have proposed it as an intermediate. SHIMIZU *et al.*[16] have shown that 20α-hydroxy-[22-^{14}C]cholesterol incubated with adrenal extracts yields [^{14}C]isocaproic acid, strongly suggesting that

the compound is converted to pregnenolone. Recently, they have demonstrated directly the formation of pregnenolone from 20α-hydroxycholesterol[17]. Thus, two precursors of pregnenolone are known. It may be seen from the data of Table VI that in the absence of Ca^{2+} exogenous cholesterol is poorly converted to pregnenolone, while exogenous 20α-hydroxycholesterol is readily converted to pregnenolone in accordance with the results of SHIMIZU *et al.*[16,17]. In the presence of Ca^{2+}, however, the conversion of exogenous cholesterol to pregnenolone is increased while that of 20α-hydroxycholesterol is actually somewhat decreased. These results indicate that the stimulation of pregnenolone formation by Ca^{2+} in pellet 2 at pH 6.2 takes place at the hydroxylation of cholesterol to 20α-hydroxycholesterol.

However, with whole homogenates, incubated under conditions where both Ca^{2+} and freezing stimulate corticoid production from endogenous precursors, it was found that the conversion of exogenous 20α-hydroxycholesterol to corticoids was stimulated 3 fold by Ca^{2+} but only slightly by freezing (Table VII). Ca^{2+} added to the frozen preparation had only a slight effect.

TABLE VI

THE EFFECT OF Ca^{2+} ON PREGNENOLONE SYNTHESIS FROM EXOGENOUS PRECURSORS

The incubation medium contained 0.3 ml of pellet 2, 90 μmoles of phosphite buffer (pH 6.2), 1.6 mg of NaTPN, 2.1 mg of glucose 6-phosphate, 0.10 unit of glucose 6-phosphate dehydrogenase and the indicated additions and 0.154 *M* KCl to a final volume of 2.0 ml. Where indicated, 60 μg of the sterols in 0.02 ml ethanol were added. The same volume of ethanol was also added to the control incubations. Incubation was for 1.0 h at 37° in air.

Ca^{2+} (mM)	*Pregnenolone synthesized (μg/beaker)*				
	no addition	*cholesterol*		*20α-hydroxycholesterol*	
			Net		*Net*
	3.27	4.09	0.8	13.2	9.9
0.55	7.22	9.80	2.6	15.8	8.6
2.75	8.27	11.4	3.1	15.6	7.3

TABLE VII

THE EFFECT OF Ca^{2+} AND FREEZING ON THE TRANSFORMATION OF 20α-HYDROXYCHOLESTEROL TO CORTICOIDS IN ADRENAL WHOLE HOMOGENATES

Each beaker contained 40 μmoles of $NaHCO_3$, 0.3 ml of the homogenate, 1.6 mg of NaTPN, 2.1 mg of Na glucose 6-phosphate, and 0.154 *M* KCl to a final volume of 2.0 ml. 60 μg of 20α-hydroxycholesterol was present where indicated. Incubation was for 1.0 h under O_2–CO_2 (95:5). The analysis for corticoids was performed according to procedures described elsewhere[1].

Homogenate	Ca^{2+} (mM)	*Corticoid synthesized (μg/beaker)*		
		no addition	*20α-hydroxycholesterol*	
				Net
Normal	—	3.8	12.3	8.5
	0.28	10.1	35.4	25.3
	0.55	11.4	35.2	23.8
	1.1	11.0	35.0	24.0
	5.5	13.0	35.5	22.5
	16.5	15.7	32.8	17.1
Frozen	—	12.1	21.9	9.8
	1.1	16.2	28.7	12.5
	5.5	17.5	29.2	11.7

Since the whole homogenate is incubated at pH 7.5, which is in the optimal range for the conversion of endogenous substrate to corticoids[1], the transformation of exogenous cholesterol and 20α-hydroxycholesterol to pregnenolone was tested as a function of pH in the pellet-2 preparation. It may be seen from the data of Table VIII that the Ca^{2+} stimulation of cholesterol to pregnenolone is seen only at low pH values while that of 20α-hydroxycholesterol to pregnenolone is seen at the higher pH values. The transformation of cholesterol and 20α-hydroxycholesterol to pregnenolone in pellet 2 as a function of Ca^{2+} concentration is seen in Table IX. The stimulation is seen at the lowest Ca^{2+} concentrations used. At higher Ca^{2+} concentrations the conversion of 20α-hydroxycholesterol to pregnenolone is diminished. This is also seen in whole homogenates (Table VII).

TABLE VIII

THE EFFECT OF pH ON THE STIMULATION BY Ca^{2+} OF THE TRANSFORMATION OF CHOLESTEROL AND 20α-HYDROXYCHOLESTEROL TO PREGNENOLONE BY PELLET 2

When present, Ca^{2+} was at 0.55 m*M* final concentration. Tris–phosphite buffers were used. Other additons and incubation conditions were the same as for Table VI.

Expt.	*pH*	*Pregnenolone synthesized (μg/beaker)*									
		—	Ca^{2+}	*Cholesterol*		*Cholesterol + Ca^{2+}*		*20α-Hydroxycholesterol*		*20α-Hydroxycholesterol + Ca^{2+}*	
					Net		*Net*		*Net*		*Net*
1	6.0	4.2	7.1	5.2	1.0	8.0	0.9				
	6.5	3.8	7.2	4.6	0.8	9.1	1.9				
	7.0	2.6	6.4	3.5	0.9	7.6	1.2				
	7.25	2.2	5.8	2.8	0.6	6.6	0.8				
	7.5	1.9	5.3	2.0	0.1	5.5	0.2				
2	7.0	2.1	7.2					12.0	9.9	16.7	9.5
	7.25	1.3	5.9					10.6	9.3	16.2	10.3
	7.5	1.3	5.1					8.7	7.4	17.1	12.0
	7.75	1.0	3.5					7.7	6.7	16.9	13.4
	8.0	0.8	2.9					6.5	5.7	13.0	10.1

TABLE IX

THE EFFECT OF Ca^{2+} ON THE TRANSFORMATION OF CHOLESTEROL AND 20α-HYDROXYCHOLESTEROL TO PREGNENOLONE IN PELLET 2

Phosphate buffer was used at pH 6.2 and Tris buffer at pH 7.5. Other additions and incubation conditions were the same as for Table VI.

Ca^{2+} (mM)	*Pregnenolone synthesized (μg/beaker)*					
	pH 6.2			*pH 7.5*		
	No addition	*Cholesterol*		*No addition*	*20α-Hydroxycholesterol*	
			Net			*Net*
—	5.08	6.58	1.5	2.21	14.7	12.5
0.16	8.46	12.5	4.0	6.77	22.5	15.7
0.55	9.84	13.7	3.9	7.16	24.5	17.3
2.2	10.4	16.2	5.8	6.64	22.8	16.2
5.5	8.98	13.0	4.0			
11.0				8.21	19.2	11.0

TABLE X

THE TRANSFORMATION OF 20α-HYDROXYCHOLESTEROL TO PREGNENOLONE BY NORMAL AND FROZEN PELLET-2 PREPARATIONS

Conditions were the same as for Table VI except where indicated.

Pellet 2	*Ca^{2+} (mM)*	*Pregnenolone synthesized (μg/beaker)*					
		pH 6.2			*pH 7.5*		
		No addition	*20α-Hydroxycholesterol*		*No addition*	*20α-Hydroxycholesterol*	
				Net			*Net*
Normal		3.50	17.9	14.4	1.20	14.3	13.1
	0.55	8.30	18.3	10.0	2.86	19.7	16.8
Frozen		0.84	1.33	0.5	1.81	2.37	0.6
	0.55	1.12	2.05	0.9	2.16	2.65	0.5

TABLE XI

THE REQUIREMENT FOR TPNH FOR THE CONVERSION OF 20α-HYDROXYCHOLESTEROL TO PREGNENOLONE BY PELLET 2

The incubation medium contained 0.3 ml of pellet 2, 90 μmoles of phosphite buffer (pH 6.2), and the indicated additions and 0.154 *M* KCl to a final volume of 2.0 ml. Where indicated 1.6 mg of NaTPN, 2.1 mg of glucose 6-phosphate, 0.10 unit of glucose 6-phosphate dehydrogenase and 60 μg of 20α-hydroxycholesterol in 0.02 ml ethanol were added. The same volume of ethanol was also added to the control incubations. Incubation was for 1.0 h at 37° in air.

Addition	*Pregnenolone synthesized μg/beaker*	
		20α-hydroxy-cholesterol
	0	0.2
TPN + glucose 6-phosphate	1.16	2.77
TPN + glucose 6-phosphate + glucose 6-phosphate dehydrogenase	4.45	14.7

As indicated in Fig. 2 the freezing of pellet 2 diminishes the formation of pregnenolone from endogenous precursors at pH 6.2 but increases it at pH 7.5. The normal pellet-2 preparation readily converts 20α-hydroxycholesterol to pregnenolone but the frozen pellet-2 preparation has very low activity in this respect at both pH values (Table X) despite the increase in pregnenolone formation at pH 7.5 from endogenous precursors. It should be noted that this increased activity of the frozen pellet 2 is better seen in a 2-h incubation than in a 1-h incubation.

The data of Table XI show that TPNH is required for the conversion of 20α-hydroxycholesterol to pregnenolone by the pellet-2 preparation.

DISCUSSION

The results presented indicate that the stimulation of corticoid synthesis by freezing and by Ca^{2+} seen in adrenal whole homogenates[1] is indeed due to the stimulation of the steps anterior to the production of progesterone as suggested earlier[2]. The

requirements for stimulation by divalent cations in pellet 2 and in the whole homogenate are qualitatively similar. The stimulation by Fe^{2+} seen in the whole homogenate system is not present here, suggesting the possibility that this cation may act at a step subsequent to pregnenolone formation. Both systems require the continuous presence of Ca^{2+}, *i.e.*, a proenzyme to enzyme transformation is probably not involved. There is a difference between the systems in that the synthesis of pregnenolone in pellet 2 is inhibited by freezing, while in the synthesis of corticoids freezing pellet 2 was stimulatory[1]. This discrepancy is, however, more apparent than real. The pH optimum for the series of reactions leading to corticoid synthesis in the whole homogenate is in the neighborhood of pH 7.5 (see ref. 1). At this pH, pregnenolone synthesis by the frozen pellet 2 is greater than that of the non-frozen preparation (Fig. 2) and would account for the stimulation of corticoid production from endogenous precursors seen in frozen whole homogenates.

The locus of the stimulation by Ca^{2+} of pregnenolone formation in pellet 2 was found to depend on the pH. At pH 6.2 Ca^{2+} stimulates the hydroxylation of cholesterol to 20α-hydroxycholesterol and actually somewhat inhibits the transformation of 20α-hydroxycholesterol to pregnenolone. At pH 7.5 Ca^{2+} stimulates the conversion of 20α-hydroxycholesterol to pregnenolone. In addition, the transformation of 20α-hydroxycholesterol to corticoids in the whole homogenate at pH 7.5 is stimulated by Ca^{2+}. The action of Ca^{2+} at two sites in the sequence of reactions from cholesterol to corticosterone could account for the Ca^{2+} concentration curve exhibiting two maxima previously observed in the synthesis of corticoids by adrenal whole homogenates[1]. Presumably, each Ca^{2+} sensitive step reacts uniquely to varying concentrations of Ca^{2+} and the Ca^{2+} concentration curve in whole homogenates would correspond to a composite of the two curves under the conditions of corticoid synthesis prevailing.

The stimulation by freezing, of corticoid synthesis in whole homogenates[1] and of pregnenolone synthesis in pellet 2 (Fig. 2), in both cases from endogenous precursors and at pH 7.5, appears to be due to the stimulation of the conversion of cholesterol to 20α-hydroxycholesterol. This conclusion is based on the following evidence. (a) At pH 7.5, freezing a whole homogenate results in an increase in corticoid production from endogenous precursors due to events occurring in pellet 2 and at a step prior to the formation of progesterone[1,2]. However, there is only a very small increase in the transformation of exogenous 20α-hydroxycholesterol to corticoids. (b) The frozen pellet-2 preparation is less effective than the normal pellet 2 in transforming exogenous 20α-hydroxycholesterol to pregnenolone, but is more effective then the normal pellet 2 in transforming endogenous precursor to pregnenolone at pH 7.5.

Several observations indicate that at pH 7.5 in the normal whole homogenate the initial step in corticoid biosynthesis from cholesterol, *i.e.*, the formation of 20α-hydroxycholesterol, is perhaps the rate-limiting step. Pregnenolone synthesis from endogenous precursors is very low at pH 7.5 in the pellet-2 preparation. However, 20α-hydroxycholesterol is readily converted to pregnenolone in this preparation at this pH. In addition, 20α-hydroxycholesterol (this paper), pregnenolone and progesterone[2] are readily converted to corticoids by whole homogenates at pH 7.5. Other evidence supporting the above conclusion is furnished by the observation that although the freezing of pellet 2 diminishes greatly the conversion of 20α-hydroxycholesterol to pregnenolone, this preparation shows a greater synthesis of pregnenolone from endogenous precursor at pH 7.5 than does the non-frozen preparation. It would appear

that in the normal pellet 2 the formation of 20α-hydroxycholesterol is rate limiting, since the stimulation of this step by freezing results in increased pregnenolone formation despite the inhibition of the transformation of 20α-hydroxycholesterol to pregnenolone.

It should be pointed out that the calculation of net synthesis in the presence of exogenous substrates and the conclusions drawn therefrom are based on the assumption that the addition of exogenous substrate does not materially affect the rate of synthesis from endogenous substrate.

ACKNOWLEDGEMENTS

I wish to thank Dr. W. WELLS and Dr. C. SWEELEY for performing the gas-chromatography analysis, and R. WINTERMYER, J. JAO and B. O'MALLEY for their competent technical assistance.

This work was supported by Grant A-3676 Endo. from the United States Public Health Service.

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