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METABOLISM OF PREGNENOLONE IN ADRENAL HOMOGENATES AT LOW TEMPERATURES

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SUMMARY

Endogenous and radioactively-labelled exogenous pregnenolone was metabolized in adrenal homogenates (0·32 M sucrose, pH 6·8) of the rabbit, the cow and the ewe when kept at temperatures between 0 and +6°C. This process could be prevented by the addition of cyanoketone, an inhibitor of the enzyme 5-ene-3β-hydroxysteroid dehydrogenase complex. The activity of the enzyme at low temperatures can lead to erroneous conclusions when studying the subcellular distribution of steroids in tissues in which it is present. No metabolism of pregnenolone was observed in dog adrenal homogenates at low temperatures.

INTRODUCTION

WHEN studying the subcellular distribution of intrinsic steroids in the adrenal gland a considerable loss of 3β-hydroxy-5-pregnen-20-one (pregnenolone) was observed in some species during the time that elapsed between homogenizing the tissue and obtaining the different fractions, although all procedures were carried out at temperatures not exceeding +6°C. This indicated that the metabolism of adrenal steroids is not completely inhibited at temperatures between +6 and 0°C. In the present paper we wish to describe some observations on this phenomenon.

METHODS

Freshly excised adrenal glands from different mammalian species were placed on watch glasses which were standing on crushed ice. The glands were dissected free from adherent connective tissue, weighed, minced with scissors, and homogenized in a teflon-glass homogenizer (clearance: 0·1–0·15 mm) using about 10 ml of ice-cooled 0·32 M sucrose per g adrenal tissue. The homogenizer was surrounded by crushed ice. Differential centrifugation was carried out in refrigerated centrifuges. The "nuclear fraction", P_1, was obtained after centrifuging the original homogenate at 900*g* for 5 min and the "mitochondrial fraction", P_2, after centrifuging the supernatant of P_1 at 12,000*g* for 30 min in a Servall centrifuge (2°C). The "microsomal fraction", P_3, and the "high speed supernatant" (S_3) were separated by spinning the supernatant of P_2 at 100,000*g* for 60 min in a Beckman Model L preparative ultracentrifuge (1°C).

The individual fractions or portions of the whole homogenate were suspended in glass distilled water (final volume: 10 ml) to lyse the particles. When intrinsic steroids were to be measured, radioactively-labelled steroids were added to enable correction for losses occurring during the further procedures. The samples were extracted with ethyl acetate (3 × 10 ml), and the extracts purified as described previously [1]. The individual steroids were separated by paper chromatography first in the E_2B system of Eberlein and Bongiovanni [2] in which pregnenolone and progesterone travelled with the solvent front and cortisol and corticosterone

were well separated. The eluate of the solvent front was re-chromatographed in the E_1 system of Eberlein and Bongiovanni[2] in which pregnenolone and progesterone were separated. The steroids were located on the chromatograms by scanning the strips for the presence of UV absorbing spots in a recording densitometer and for the presence of radioactivity in a Packard Radiochromatogram scanner. Cortisol and corticosterone were estimated by their reaction with blue tetrazolium, and pregnenolone and progesterone by gas–liquid chromatography (3·8% SE-30 columns, flame ionization detection) using 11βOH-progesterone as internal standard[1].

RESULTS

The first observations were made on the homogenate of the adrenal glands of a ewe (77·5 kg); the glands were extirpated under sodium pentobarbitone anaesthesia. Of this homogenate, 20% was extracted immediately; the rest was subjected to differential centrifugation. The results (Table 1) show that 45% of the pregnenolone contained in the original homogenate disappeared during the centrifugation procedures whereas the amounts of cortisol and progesterone found after centrifugation were larger than expected. Expressed on a molar basis the amounts of progesterone plus cortisol gained were about the same as the amount of pregnenolone lost. This suggests, that pregnenolone was metabolized at the low temperatures at which the samples were kept during centrifugation.

Consequently an experiment was carried out to test whether pregnenolone added to minced adrenal tissue before homogenization is converted to other steroids under the conditions under which subcellular fractionation is carried out. The freshly excised adrenal glands (1·3g) from 3 female rabbits were placed on an ice cooled watch glass, minced with scissors in a cold room (5°C), and transferred to a homogenizer surrounded by crushed ice; [4-^{14}C]pregnenolone (0·25 μCi in 150 μl of ethanol) was added followed by 2·5 ml of cold 0·32 M sucrose (pH 6·8). The tissue was homogenized and the homogenizer washed several times until the final volume of the homogenate was 12·5 ml. The temperature of the homogenate at the end of homogenization was 4°C. It was well mixed by shaking, 2·5 ml were removed, diluted with an equal volume of water

Table 1. Conversion of pregnenolone in a sheep adrenal homogenate (0·32 M sucrose, pH 6·8) during differential centrifugation in the cold

Sample	Steroid content (n-moles) Pregnenolone	Progesterone	Cortisol
S_I: Total homogenate before centrifugation*	169	5	116
S_{II}: Sum of each steroid found in the different fractions†	93	58	141
Difference between S_I and S_{II}	−76	+53	+25

*Calculated from the estimates obtained in 20% of the homogenate.

†Corrected for the 20% withdrawn from the original homogenate.

and extracted with ethyl acetate. The time that elapsed between adding the [4-^{14}C]-pregnenolone and the extraction of this first sample (S_1) was 15 min. The remainder of the homogenate was subjected to differential centrifugation. The P_1 fraction was extracted about 45 min after the addition of the [^{14}C]-pregnenolone, the P_2 fraction after about 120 min and the P_3 fraction and high speed supernatant after about 300 min. In addition, a "control sample" was prepared which consisted of 20 μg unlabelled pregnenolone and 0·033 μCi [4-^{14}C]-pregnenolone in 2·5 ml 0·32 M sucrose. This "control sample" was extracted immediately and treated in the same fashion as the biological samples.

Figure 1 shows the radioactivity scans of the E_1 chromatograms (1 cm lanes) of several samples analysed in this experiment. The bottom tracing was obtained from the control sample, which did not contain any biological material. Only one peak of radioactivity in the pregnenolone region was visible. In sample 1, the extract of 20 per cent of the total homogenate which was obtained 15 min after the [^{14}C]-pregnenolone came into contact with adrenal tissue, there was in addition to the pregnenolone peak, a peak in the region corresponding to progesterone. The progesterone peak was far more pronounced in the scan of the P_1 extract. In addition, this sample showed two peaks of radioactivity near the origin which were not identified. The extracts of the P_2 and P_3 fractions showed scans very similar to that of the P_1 fraction, the pregnenolone and progesterone peaks being somewhat smaller. The extract of the third supernatant (S_3) showed only a small peak in the pregnenolone region and two peaks near the origin. There was also some radioactivity associated with corticosterone. No conversion of pregnenolone occurred in the sample which did not contain adrenal tissue.

The time course of the disappearance of pregnenolone in a rabbit adrenal homogenate kept at 6°C is shown in Fig. 2. To the well cooled homogenate

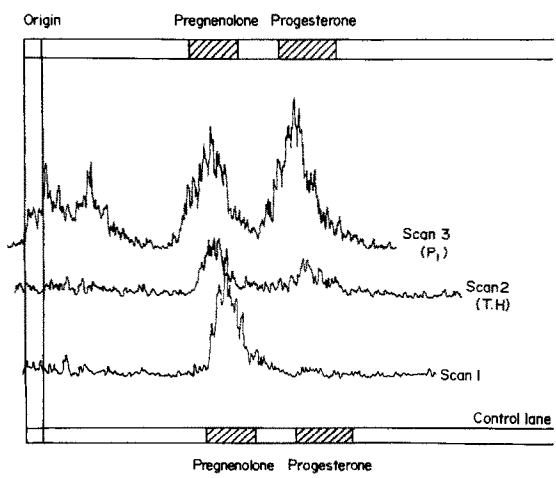

Fig. 1. Conversion of pregnenolone to progesterone in the course of the subcellular fractionation of a rabbit adrenal homogenate (pH 6·8). [4-^{14}C]-pregnenolone (0·25 μCi) was added to the homogenate (= time 0). Radioactivity scans of E_1 chromatograms [2] (1 cm lanes separated by 0·5 cm spaces) of different samples. Scan 1: Extract of 2·5 ml 0·32 M sucrose to which 20 μg unlabelled pregnenolone (carrier) and [4-^{14}C]-pregnenolone (0·033 μCi) had been added. Scan 2: 20 per cent of total homogenate (T.H.) extracted 15 min after the addition of the radioactive pregnenolone. Scan 3: Extract of P_1 fraction (T.H. centrifuged at 900g for 5 min at 2°C), extracted about 45 min after S_1.

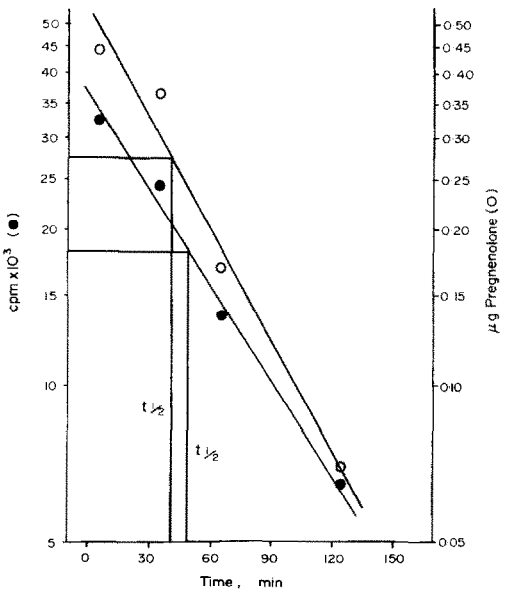

Fig. 2. Calculated regression lines for the metabolism of pregnenolone in a rabbit adrenal homogenate (0·08g tissue/ml 0·32 M sucrose, pH 6·8) kept at 5–6°C; [4-^{14}C]-pregnenolone (0·6 μCi) was added to the total homogenate (12 ml) at time = 0. Three ml samples (= 0·24 g adrenal tissue/sample) were removed for steroid extraction at 5, 35, 66 and 126 min after the addition of radioactive pregnenolone. Observed values: ○ = endogenous pregnenolone (μg/0·24 g tissue; estimates not corrected for losses); ● = radiocounts found in the eluates of the pregnenolone regions of the E_1 chromatograms of the sample extracts. $t_{1/2}$ = half life time. ○: $t_{1/2}$ = 41 min; ●: $t_{1/2}$ = 48·8 min.

(0·08 g/ml) of 8 rabbit adrenal glands [4-^{14}C]-pregnenolone (0·05 μCi/ml) was added, well mixed and 3 ml of the homogenate extracted 5 min later. The remainder was left in the refrigerator at 5–6°C. Further portions of 3 ml were extracted 35, 66 and 126 min later. The purified extracts were chromatographed and the pregnenolone and progesterone regions eluted; 10 per cent of the eluates were used for counting the radioactivity which derived from the initially added [^{14}C]-pregnenolone, the remainder of each eluate was assayed for its content of intrinsic steroids by g.l.c. When the logarithms of the pregnenolone concentrations were plotted against time a straight line was obtained indicating a first order reaction. The velocity constants k for the extrinsic [^{14}C]-pregnenolone ($k = 0{\cdot}0142\ min^{-1}$) and the intrinsic pregnenolone ($k = 0{\cdot}0169\ min^{-1}$) were of the same order and thus their half life times were similar. As shown in Fig. 3, there was a gain in both endogenous and in [^{14}C]-progesterone after 35 min. During the following 90 min, however, progesterone was lost at a rate similar to that of pregnenolone. Unlabelled and labelled pregnenolone were also added to a sample of sucrose and allowed to stand for 2 h at 6°C. No significant loss of pregnenolone occurred and, consequently, no progesterone was formed.

Figure 4 shows the results of experiments on cow adrenal tissue. To a homogenate of a cow adrenal gland containing cortical and medullary tissue (0·11 g/ml) [4-^{14}C]-pregnenolone (0·01 μCi/ml) was added. The homogenate was kept at 0°C and its content of [^{14}C]-pregnenolone estimated at different times after the addition. In contrast to the rabbit adrenal homogenate which was kept at 6°C the dis-

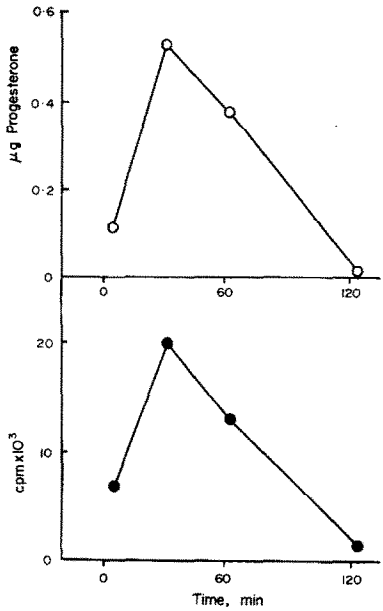

Fig. 3. Changes in the content of endogenous (○—○) and [^{14}C]-progesterone (●—●) in the samples of the same rabbit adrenal homogenates as in Fig. 2. All [^{14}C]-progesterone was formed from [^{14}C]-pregnenolone added at time 0 (see text and legend to Fig. 2).

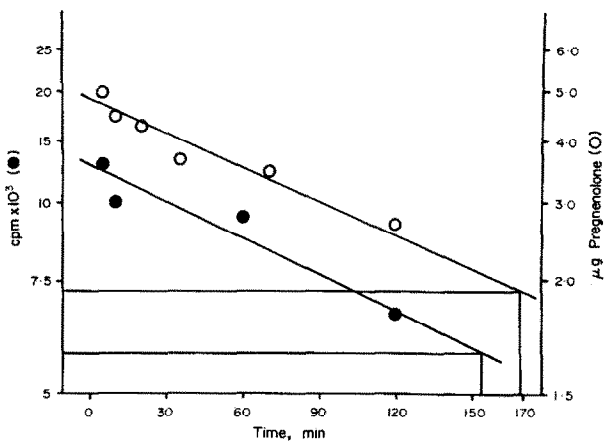

Fig. 4. Calculated regression lines for the metabolism of pregnenolone in cow adrenal homogenates kept surrounded by crushed ice at 0°C (temperature at end of homogenization: +4°C, after 10 min: 0·5°C. After 30 min: 0°C). Observed values: ○ = Endogenous pregnenolone (μg/5 g tissue) in the homogenate of cortical tissue from a cow adrenal gland (0·1 g/ml 0·32 M sucrose, pH 6·8, 5 ml samples analysed); ● = [^{14}C]-pregnenolone (0·01 μCi/ml) added to a homogenate of cortical and medullary tissue of a cow adrenal gland (0·11 g/ml 0·32 M sucrose, pH 6·8, 2 ml samples analysed). $t_{1/2}$: Half life time. ○ $t_{1/2}$ = 169 min; ● $t_{1/2}$ = 154 min.

appearance rate of pregnenolone in the cow adrenal homogenate kept at 0°C was slower, the half life time being 154 min. The endogenous pregnenolone was estimated in the homogenate of adrenal cortical tissue (0·1 g/ml) of the second cow and the results are included in Fig. 4. The half life time of the endogenous pregnenolone was similar to that of the extrinsic compound.

Figure 5 shows the results of an experiment on a cow adrenal homogenate (0·11 g/ml) to which [^{14}C]pregnenolone (0·01 μCi/ml) had been added. Two control samples were removed at time 0. The remainder was divided into two portions. One was kept at 0°C, the other at 37°C. The figure shows the very fast disappearance of pregnenolone at 37°C when compared with that at 0°C.

Table 2 summarises the results of similar experiments on rabbits, cow and dog

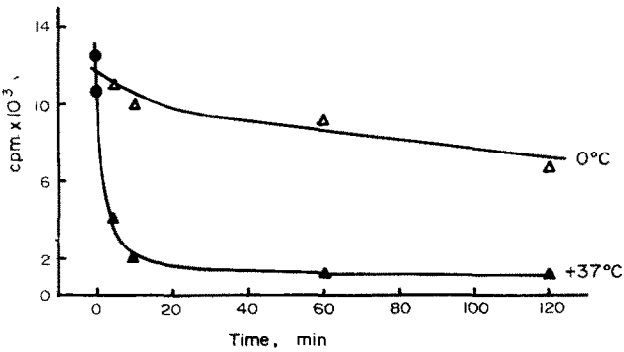

Fig. 5. Metabolism of [^{14}C]-pregnenolone (0·01 μCi/ml) in a cow adrenal homogenate (0·11 g tissue/ml). One half (△) was kept at 0°C, the second (▲) at 37°C; 2 ml portions were extracted at the times indicated ($t_{1/2}$ at 37°C: 5 min). ● = samples taken at time zero.

Table 2. Loss of pregnenolone in adrenal homogenates (0·32 M sucrose, pH 6·8) at low temperatures

Species	Type of pregnenolone measured	Temperature (°C)	Incubation time (min)	Pregnenolone content in % of pregnenolone present at time "0"	Mean half life times (min)
Rabbit	Endogenous pregnenolone	+3	60	45	
	Endogenous pregnenolone	+6	60	39	
	Endogenous pregnenolone	+6	120	16	50 ± 3·2
	[4-^{14}C]-pregnenolone	+6	60	42	
	[4-^{14}C]-pregnenolone	+6	120	26	
	[4-^{14}C]-pregnenolone	0*	240	51	
	[4-^{14}C]-pregnenolone	0*	240	51	252 ± 25·7
	[4-^{14}C]-pregnenolone	0*	240	55	
	[4-^{14}C]-pregnenolone	0*	240	40	
Cow	Endogenous pregnenolone	0*	70	84	
	Endogenous pregnenolone	0*	120	69	
	[4-^{14}C]-pregnenolone	0*	120	58	203 ± 21·0
	[4-^{14}C]-pregnenolone	0*	120	69	
	[4-^{14}C]-pregnenolone	0*	60	79	
	[4-^{14}C]-pregnenolone	0*	120	57	
Dog	Endogenous pregnenolone	0*	180	108, 112	
	Endogenous pregnenolone	0*	180	101, 106	

The time regarded as "0" was either 0·5–1·0 min after finishing the homogenization of the adrenal tissue or 0·5–1·0 min after the addition of [^{14}C]-pregnenolone.

*Temperature at end of homogenization: 4°C, after 10 min it was 1°C, after 30 min: 0°C. The pH of the homogenates remained constant.

adrenal homogenates. In rabbit adrenal homogenates kept at 0°C the half life time of pregnenolone was about $\frac{1}{5}$th of that in homogenates kept between 3–6°C. In cow adrenals kept at 0°C the half life time of pregnenolone was similar to that in rabbit adrenal homogenates at 0°C. In the homogenates of dog adrenal glands kept at 0°C no loss of pregnenolone was observed.

To obtain further evidence for the enzymatic nature of the disappearance of pregnenolone in adrenal homogenates at low temperatures cyanoketone (2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one), an inhibitor of the 5-ene-3β-hydroxy steroid dehydrogenase [3, 4], which is required for the transformation of pregnenolone to progesterone, was added to the adrenal homogenates. The results in Table 3 show that cyanoketone inhibited the transformation of pregnenolone.

Table 3. Effect of cyanoketone (CK) on the disappearance of pregnenolone in rabbit adrenal homogenates at 0°C

Experiment	Sample		Incubation time at 0°–+1°C (min)	d.p.m. associated with pregnenolone (%)*
1	Control	S_1, S_2	1	100
	CK	S_3	240	101
	added	S_4	240	97
	No CK	S_5	240	51
	added	S_6	240	57
2	Control	S_1	1	100
	CK added	S_2	240	88
	No CK added	S_3	240	55

In both experiments the homogenates contained 19 mg tissue in 1 ml 0·32 M sucrose (pH 6·8). CK: 1 μg/2 ml homogenate. 200 μg of cold pregnenolone (as carrier) and 50 μCi [4-^{14}C]-pregnenolone were added at time 0.

*The d.p.m. found in the pregnenolone region of the E_1 chromatograms of the control samples extracted 1 min after the addition of the unlabelled and labelled pregnenolone were taken as 100 per cent.

The metabolism of pregnenolone in rabbit adrenal homogenates at low temperatures could also be inhibited by using sucrose at pH 3 (50 ml 0·44 M sucrose, 40 ml 0·1 M citric acid, 10 ml 0·2 M Na_2HPO_4). Before extraction with ethyl acetate the samples were neutralized. In one experiment, 1·01 μg of pregnenolone were found in 4 ml of the freshly prepared homogenate and 1·18 μg after standing at 0°C for 2 h. In a similar experiment on a rabbit adrenal homogenate at pH 3 the amounts of pregnenolone found in two 2 ml samples were 1·8 μg and 1·7 μg at "time 0" and 1·64 and 1·7 μg after 4 h at 0°C.

DISCUSSION

When homogenates of adrenal tissue from rabbits, cows and a ewe were kept at temperatures between 0 and 6°C (pH 6·8) a loss of endogenous and extrinsic, [^{14}C] labelled pregnenolone was observed. That this loss was due to an enzymatic conversion of the steroid is suggested by the formation of radioactive progesterone from radioactive pregnenolone. Furthermore, cyanoketone, an inhibitor of the enzyme 5-ene-3β-hydroxysteroid dehydrogenase[3, 4] was able to prevent the conversion. The enzyme activity in adrenal homogenates containing about 100 mg tissue/ml is very high when kept at 37°C ($t_{1/2}$ of pregnenolone 5 min, Fig. 5). The observed $t_{1/2}$ at 5°C shows that the reaction followed the general rule that an increase in the reaction temperature of 10°C doubles the rate of the reaction. Pregnenolone was not metabolized in dog adrenal homogenates kept at low temperatures under the same conditions.

The metabolism of pregnenolone at low temperatures can lead to wrong conclusions in the study of the subcellular distribution of steroids in homogenates of adrenal glands and other tissues containing 5-ene-3β-hydroxysteroid dehydrogenase. Thus, Fig. 1 demonstrates that some of the progesterone found in the P_1 fraction was derived from pregnenolone during the processing of the homogenate of rabbit adrenal glands. As the pregnenolone concentration in the adrenal glands is usually higher than that of progesterone this can cause large errors.

Residual enzyme activities at low temperatures may also affect experiments on the subcellular distribution of substances other than steroids.

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