dexamethasone dose was 2.0 mg/6 hours may be partly reflecting drug excretion in the urine.

The present work suggests that the transitory adrenal hyperactivity occurring during exposure to the hypoxia and other stimuli present at this level of altitude can be either increased or depressed. Such results are of interest when we consider the possibility that the adrenals may play a role in improving tolerance to acute oxygen deficiency.

Summary. The response to ACTH stimulation and to dexamethasone inhibition was studied in young adult males acutely exposed to an altitude of 14,000 feet. The results indicate that the adrenal cortex can be further stimulated with exogenous ACTH during the first day of exposure to an environment of low oxygen tension. Dexamethasone administration was started the day before the trip to high altitude and depressed the excretion of urinary steroids despite the hypoxic stimulus.

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Enzyme Activities in the Preincubation Media from Adrenal Tissue Incubations.* (31318)

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The preincubation period was first introduced by Saffran and Bayliss(1), who noted that after preincubation adrenal slices produced less corticoids than the non-preincubated tissue in the absence of ACTH. Later, Schönbaum *et al*(2) reported the detection of NADP-dependent dehydrogenases in the incubation or preincubation medium from rat adrenal quarters. Recently we have obtained evidence that the preincubation media from bovine or rat adrenal tissue are capable of metabolizing added labelled pregnenolone or progesterone. These findings may have considerable interest with regard to steroidogenesis *in vitro*.

Material and methods.‡ Fresh bovine adrenal glands obtained at a local abattoir were transported to the laboratory in ice-cold Krebs-Ringer bicarbonate solution (pH 7.4) containing 200 mg% glucose (KRBG). After removal of surrounding fat, the method of Stachenko and Giroud(3) was followed to separate the zona glomerulosa (Z.G.) from the zona fasciculata (Z.F.) with the Stadie-Riggs microtome. Slices of the whole cortex were also used. In the latter case, the gland was cut at right angles to its surface, and the

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‡ ^{14}C- or 3H-labelled precursors were obtained from New England Nuclear Corp. NAD (nicotinamide adenine dinucleotide, NADP (nicotinamide adenine dinucleotide phosphate, monosodium salt), G-6-P (glucose 6-phosphate, disodium salt) were purchased from Sigma Chem. Co. Other trivial names used in the text: Pregnenolone (3β-hydroxy-pregn-5-en-20-one); 11β hydroxyprogesterone or 11β-OH-P (11β-hydroxy-pregn-4-ene-3,20-dione); 11-deoxycorticosterone or DOC (21-hydroxy-pregn-4-ene-3,20-dione); 17α-hydroxyprogesterone or 17α-OH-P (17α-hydroxy-pregn-4-ene-3,20-dione); 11-keto-progesterone (pregn-4-ene-3,11,20-trione), compound S (17α, 21-dihydroxy-pregn-4-ene-3,20-dione).

medullary tissue was removed. Slices were about 0.3 mm thick and 15 mm long.

Rat adrenal glands were obtained from male Sprague-Dawley rats weighing 150-180 g. The animals were anaesthetized with 12-18 mg of sodium pentobarbital given intraperitoneally. Adrenals were cleared of fat. Quartered or whole adrenal glands were kept in cold KRBG until used, which was within 2 hours.

The preincubation media (PIM) were prepared as follows: Bovine or rat adrenal tissue was washed 3 times with 10 ml KRBG, dried gently with filtered paper and weighed. Each preincubation contained 1.5 g of bovine cortical slices or 400-450 mg of rat adrenal quarters or whole adrenal glands (capsule intact) in 15 ml KRBG. Preincubation was done in a Dubnoff Metabolic Incubator shaking at approximately 65 cycles per minute for one hour at 38°C under 95% O_2-5% CO_2 unless otherwise stated. At the end of the preincubation period, the PIM were decanted from the tissue, pooled together into a flask and swirled to ensure uniform mixing. Fifteen ml of PIM were then incubated for 3 hours under the same conditions as described above for the preincubation period, with added ^{14}C- or ^{3}H-labelled progesterone or pregnenolone in the absence or presence of NADP (0.83 μmole/ml) G-6-P (2.53 μmoles/ml), or NAD (0.39 μmole/ml). Concentrations of substrates will be given under *Results*. Incubation products were extracted 3 times with 2 volumes of freshly distilled ethyl acetate and chloroform (1:1). The extract was dehydrated over anhydrous sodium sulfate and taken to dryness under reduced pressure at 50°C. The dried residue was redissolved in 10 ml methanol, and aliquots were taken for measurements of total radioactivity as described previously(4). The steroids were initially separated by paper chromatography in the Bush B_5 system(5) for approximately 4 hours as shown in Fig. 1. Areas in the position of cortisol or aldosterone were rechromatographed in the Bush C system(5) for about 4 hours; that with the mobility of corticosterone was rechromatographed in the Eberlein-Bongiovanni (E_2B) system(6) for 4-5 hours, in which corticosterone was separated from compound S. The solvent-front fraction in Bush B_5 contained mainly the unaltered substrates (progesterone or pregnenolone), DOC and/or 11β-hydroxyprogesterone. This fraction was rechromatographed in the ligroin-propylene-glycol (L.P.G.) system(7) for 6-8 hours, in which progesterone and/or pregnenolone were well separated. Since DOC and 11β-hydroxyprogesterone were still not separated this fraction was reapplied in the same system for 50 hours. Under these conditions DOC and 11β-hydroxyprogersterone were well separated. The latter compound was further purified in the Bush A system(5) for 4 hours. After these different chromatographic purifications, the compounds mentioned above were considered reasonably pure and aliquots were taken for counting. Further tests to confirm the identity of these compounds included: i) Porter-Silber (PS) reaction(8), and tetrazolium chloride reduction(9); ii) formation of acetylated or oxidative derivatives following the methods suggested by Bush(10), and running the derivatives in the appropriate systems with the corresponding authentic compounds; iii) ultraviolet light absorption spectrum between 230 and 260 mμ, using ethanol

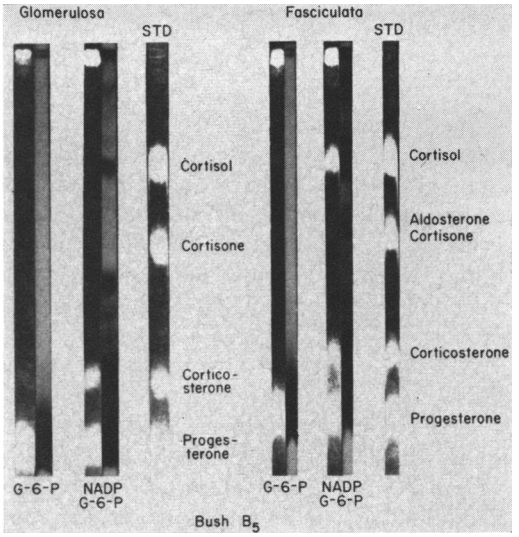

FIG. 1. Conversion products from progesterone-4-^{14}C by PIM (bovine adrenals). STD = reference standards (UV photograph). Left strip = UV photograph. Right strip = corresponding radioautograph.

as the solvent; iv) specific activity determination after crystallization of isolated radioactive 11β-hydroxyprogesterone with the authentic compound.

Results expressed as per cent conversion/incubation/3 hours were calculated by

$$\frac{\text{Radioactivity of isolated product}}{\text{Total radioactivity recovered in first extract}} \times 100.$$

As losses which occurred to the extent of 10-15% during extraction and 20-25% during each paper chromatography were not corrected for, the figures presented below must be interpreted as relative rather than absolute.

Results and discussion. A. Bovine adrenal PIM. *Incubation with progesterone-4-^{14}C.* The results are presented in Fig. 1. Hydroxylation of added progesterone-4-^{14}C (0.18 μmole; 1 μc) occurred in the presence of exogenous NADP and G-6-P. With G-6-P added alone, there was no conversion. The per cent conversion of the added radioactive precursor to the various metabolites is as follows: a) by PIM from Z.F.: corticosterone, 17.5% and cortisol, 14%; b) by PIM from Z.G.: corticosterone, 18.5% and cortisol, 0.76%. These results indicate that the 11β-17α- and 21-steroid hydroxylase activities were present in the PIM. We had also observed that NADP added alone (not shown in Fig. 1) was sufficient to effect hydroxylations, but the conversion of added labelled progesterone to corticosterone was only half that when both NADP and G-6-P were added. From this observation, it was inferred that G-6-P-dehydrogenase activity was also present in the PIM. It may be of interest that under no circumstances were we able to detect the formation of radioactive aldosterone even by PIM from the Z.G.

Incubation with labelled pregnenolone. There was very little conversion of pregnenolone-7α-^{3}H (0.16 μmole; 14 μc) to progesterone (6.6%) when this substrate was incubated with PIM from the Z.G. in the absence of NAD (Table I). However, when NAD was added, the conversion to progesterone (63.9%) was markedly increased. This would indicate the presence of Δ^5-3β-hydroxysteroid dehydrogenase and Δ^5-Δ^4 isomerase activities.

TABLE I. Conversion of Pregnenolone-17α-^{3}H by PIM (Bovine Adrenal Cortex). Each incubation contained: PIM (15 ml); pregnenolone-7α-^{3}H (0.16 μmole; 14 μc); NAD, where indicated (0.39 μmole/ml). Other incubation conditions are described in *Methods.*

Products	No NAD	NAD
	⌜—% Conversion—⌝	
Progesterone	6.6	63.9
17α-OH-P and DOC	.0	3.7
Unconverted pregnenolone	71.9	8.1

Further hydroxylations of the formed progesterone did not occur to any significant extent without addition of NADP. In another experiment (not shown in Table I) in which pregnenolone-4-^{14}C (0.17 μmole; 0.5 μc) was used, it was found that in the presence of NADP and G-6-P, 11.4% of the added substrate was converted to progesterone and 10% to corticosterone, whereas in the presence of NAD and G-6-P, 52% of the added substrate was converted to progesterone. These results seem to agree well with the observation by Kowal, Forchielli, and Dorfman(11) that NADP can partially replace NAD as the exogenous co-factor of Δ^5-3β-hydroxysteroid dehydrogenase.

Enzyme activities vs *time of preincubation.* Fig. 2 indicates the incubation conditions as

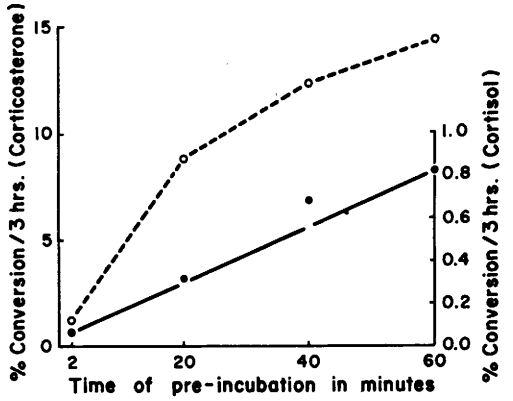

FIG. 2. Enzyme activities *vs* time of preincubation. In this experiment, 1.5 g of bovine adrenal cortical slices in 15 ml Krebs-Ringer bicarbonate glucose solution were preincubated under conditions described in *Methods* for 2, 20, 40 and 60 minutes. The corresponding preincubation media were incubated with equal amounts of progesterone-4-^{14}C (0.17 μmole; 0.5 μc), NADP (0.83 μmole/ml), and G-6-P (2.53 μmole/ml), for 3 hours under conditions as described in *Methods.* ------Corticosterone. ——— Cortisol.

TABLE II. Conversion of Progesterone-4-^{14}C by PIM (Rat Adrenal Quarters). Each incubation mixture contained: Progesterone-4-^{14}C (0.19 μmole; 1.5 μc); NADP (0.83 μmole/ml); G-6-P (2.53 μmole/ml); PIM (15 ml), prepared from an equivalent of 450 mg of rat adrenal quarters preincubated for 1 hr. Incubation conditions were described in *Methods*.

Products	% Conversion/incubation/3 hr
11β-OH-P	23.1
Corticosterone	18.9

well as the results of the effects of time of preincubation on enzyme activities. The PIM taken at the beginning of the preincubation period had little enzyme activities. These, however, increased with time of preincubation over 2 to 60 minutes, suggesting that the enzyme-containing materials actually "leaked out" into the media during the course of the preincubation period.

B. Rat adrenal PIM. *Incubation with progesterone-4-^{14}C.* When the PIM from rat adrenal quarters were incubated with labelled progesterone (0.19 μmole; 1.5 μc) in the presence of NADP and G-6-P, two major conversion products were formed, namely, 11β-hydroxyprogesterone (23.1%), and corticosterone (18.9%) (Table II).

The high yield of 11β-hydroxyprogesterone may be due to the sensitivity of 11β-hydroxylase to the NADPH-generating system, as previously shown by Makoff *et al*(12) in rat homogenate, or to the presence of a higher proportion of this enzyme in the rat PIM. These and other possibilities are being investigated.

C. Effect of temperature on preincubation. Results are given in Table III. When whole (uncut) rat adrenal glands were preincubated at 38°C the PIM failed to metabolize labelled progesterone to any appreciable extent. In the conversion of 11β-hydroxyprogesterone, the only detectable product formed was only 0.69%. However, rather unexpectedly, when the whole glands were preincubated at 4°C in a cold room, enzyme activities in the PIM were markedly increased. 11β-hydroxyprogesterone and corticosterone formed from added labelled progesterone were 6.9% and 0.69%, respectively. The same effect of low temperature preincubation on enzyme activities has also been demonstrated, using bovine cortical slices. These results are also indicated in Table III. This phenomenon is not understood. It is quite possible that low temperature would prevent enzymes from degeneration. However, Koritz and Péron reported(13,14) that there was a tremendous increase in corticoid production in pre-frozen adrenal slices or even homogenates in response to NADPH-generating system stimulation.

D. Characterization of isolated products.

a) Cortisol: i) PS reaction and tetrazolium reduction positive; ii) identical mobilities with the authentic compound in Bush B_5 and Bush C systems; iii) acetylated derivative behaving exactly as authentic cortisol-21-acetate in the toluene-propylene-glycol system (TPG)(15) and iv) maximal U.V. absorption at 240 mμ.

b) Corticosterone: i) PS reaction negative but tetrazolium reduction positive; ii) identical mobilities with the authentic compound in Bush B_5 and E_2B systems; iii) acetylated product behaving exactly as authentic corticosterone-21-acetate in the TPG system and iv) maximal U.V. absorption at 240 mμ.

c) Progesterone (conversion product from labelled pregnenolone): i) both PS and tetrazolium negative; ii) identical mobility with authentic progesterone in the LPG system,

TABLE III. Effect of Temperature on Preincubation. Rat adrenal PIM were prepared by preincubating 400 mg whole gland with 15 ml Krebs-Ringer bicarbonate glucose solution (KRBG) for 1½ hr. Bovine adrenal PIM were prepared by preincubating 1.5 g tissue from the Z.G. with 15 ml KRBG for 1 hr. Temperatures of the preincubation period are indicated in the Table. Subsequent incubations of PIM were carried out at 38°C for 3 hr in the presence of NADP and G-6-P as indicated in Fig. 2. In experiments (A) 0.17 μmole (1 μc) and (B) 0.18 μmole (1 μc) of progesterone-4-^{14}C were added.

		% Conversion/incubation/3 hr	
		(A) PIM	(B) PIM
Products	Preincubation temp	Rat adrenal (whole gland)	Bovine adrenal (Z.G.)
Corticosterone	38°C	.0	18.5
	4°C	.86	51.4
11β-OH-P	38°C	.69	—
	4°C	6.90	—

TABLE IV. Crystallization of 11β-Hydroxyprogesterone. About 5 μg of isolated 11β-hydroxyprogesterone, which had previously been purified in the ligroin-propylene-glycol and Bush A systems and filtered through a small sintered glass funnel were mixed with 10 mg of the non-radioactive authentic compound. Crystallization was carried out in 3 different solvents, methanol, benzene, and ethanol. In each case, the material was first dissolved in minimal amount of acetone.

Crystallization	Solvents	cpm/mg Crystals	cpm/mg Mother liquors
First	Acetone-methanol	11.98×10^3	8.42×10^3
Second	Acetone-benzene	11.55×10^3	11.95×10^3
Third	Acetone-ethanol	12.22×10^3	11.95×10^3

and iii) maximal U.V. absorption at 240 mμ.

d) 11β-hydroxyprogesterone: i) both PS and tetrazolium reactions negative; ii) identical mobilities with the authentic compound in the LPG and Bush A systems; iii) oxidative product inseparable from authentic 11-keto-progesterone in the LPG and Bush A systems; iv) not acetylatable with acetic anhydride and pyridine; v) constant specific activity being attained after repeated crystallization with the authentic compound (Table IV).

Our results have the following significance: 1) The recognition of enzyme activities in the media raises the possibility that the incubation media may play an important role in the metabolism of steroid in tissue incubation *in vitro,* particularly where co-factors and substrates are added. In this connection, it may be of interest that addition of G-6-P and NADP to the incubation medium containing adrenal sections markedly increased the endogenous production of corticoids(13), in spite of the fact that G-6-P, and presumably NADP or NADPH, cannot penetrate the intact cell membrane of other tissues as well as yeast cells(16,17). As mentioned above, G-6-P-dehydrogenase has been found in the PIM.

2) Loss of enzyme activities to the PIM may in part explain the earlier observation by Saffran and Bayliss(1) that there was a reduced production of total corticoids in rat adrenal quarters which were preincubated as compared to those which were not. It is not clear from these experiments whether or not these enzymes detected in the media are associated with the sub-cellular particles.

Summary. The preincubation media, consisting of Krebs-Ringer bicarbonate glucose solution, in which well washed bovine or rat adrenal slices or sections have been incubated, have the capacity to metabolize labelled progesterone or pregnenolone when supplemented with the appropriate co-factors. The 17α-, 11β-, and 21-hydroxylase activities are activated by exogenous NADP and G-6-P, whereas the enzyme systems involved in the transformation of pregnenolone to progesterone are apparently NAD- or NADP-dependent. Enzyme activities increase in the media as time of preincubation is prolonged. Preincubation at low temperature seems to increase enzyme activities.

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