Adrenal Corticosteroidogenesis and Hypothyroidism: Effect of Long Term Treatment with P-Aminobenzoic Acid*

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The relationship between induced hypothyroidism and adrenal involution has been studied by a variety of approaches in an attempt to elucidate the physiological basis between the events. McCarthy *et al.* (1959) reported on an investigation of feeding several antithyroidal agents to rats to study adrenal gland involution. While adrenal involution did occur following treatment with several of the goitrogens, only in one case was there a difference in peripheral plasma adrenal corticoid levels. In rats fed p-aminobenzoic acid (PABA) for 12 weeks, peripheral levels of corticosterone[1] (B) decreased and levels of a Porter-Silber positive chromogen increased markedly. Work from the laboratories of Peron (1961) and of Birmingham (1961) established that the Porter-Silber chromogen in rat blood is 18-hydroxydeoxycorticosterone (18-OH DOC). Cortes and Peron (1964) compared adrenal venous effluent of normal and PABA-tested rats. These workers concluded that adrenal venous secretion of B and of 18-OH DOC by PABA-treated rats was unchanged from control values.

Since the level of PABA fed has been shown to decrease thyroid function (McCarthy and Murphree, 1960), a question arose as to whether adrenal tissue from these hypothyroid rats would show any modification in corticosteroidogenesis. This study was undertaken to examine adrenal corticoid conversion in adrenal glands from rats fed PABA for various periods of time.

MATERIALS AND METHODS

Male Holtzman rats, 130 to 140 grams body weight, were housed 5 to 10 animals per colony cage under conditions of controlled light (14 hours light - 10 hours dark) and temperature (72°F). All animals

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[1] The systematic nomenclature of compounds for which trivial names are used in this report are: Corticosterone (B), 11β, 21-dihydroxy-4-pregnen-3,20-dione; 18-hydroxy-deoxycorticosterone (18- OHDOC), 18, 21-dihydroxy-4-pregnen-3, 20-dione; pregnenolone, 3-hydroxy-5-pregnen-20-one; progesterone, 4-pregnen-3, 20-dione; 11-deoxycorticosterone, 21-hydroxy-4-pregnen-3, 20-dione; G-6-P, glucose-6-phosphate.

were given tap water to drink *ad libitum*. Control rats were fed ground Purina rat food while experimental animals received the ground food containing 2% or 4% PABA by weight. For each experiment, 14 to 18 rats were used for each control or treatment group.

Rats were treated for periods of 2 to 12 weeks then killed by decapitation. Adrenal glands were rapidly removed and stored on filter paper moistened with 0.25 M sucrose and maintained in a cold chamber. The glands were cleaned of adhering fat, weighed, then homogenized in 0.25 M sucrose. In the first experiments, amounts of homogenates equivalent to 20 mg wet weight adrenal gland per 0.3 ml homogenate were incubated in $NaHCO_3$ buffer 20 mM, with KCl 7.7 mM, 800 μg reduced nicotinamide adenine dinucleotide phosphate (NADPH) or an NADPH generating system (glucose-6-phosphate, 2.1 mg and NADP 1.8 mg). Pregnenolone or progesterone 60 μg in 0.02 ml of an ethanol-propylene glycol mixture 1:1 was added as precursor steroid. Total volume of the incubation mixture was brought to 2.0 ml with 0.154 M NaCl.

In other experiments, 11-deoxycorticosterone (DOC) was added as precursor corticoid to examine 11β-hydroxylation reactions. Incubations were carried out in a manner as outlined above except that the buffer was Tris 20 mM. The conversion of DOC into corticosterone (B) was supported by adding NADPH 800 μg and calcium ions (Ca^{2+} 11 mM) or by oxidizable intermediates such as malate, isocitrate, etc. 10 mM.

All incubations were carried out at 37°C in a Dubnoff metabolic incubator for 30 to 60 minutes. Steroid analyses were carried out on dichloromethane extracts of aliquots of the incubation media. Conversion of pregnenolone or progesterone to α-ketols was measured by the blue tetrazolium method of Elliott *et al.* (1954). Corticosterone determinations were carried out fluorometrically using the method of Moncloa *et al.* (1959).

RESULTS

The first experiments concerned evaluating the effects of length of PABA treatment on the ability of adrenal homogenate preparations to convert pregnenolone or progesterone into corticoid product (Table 1). These studies were carried out in the absence of Ca^{2+}, hence the primary α-ketol product was DOC. The administration of 2% PABA to rats for 10 weeks had little effect to alter the conversion of precursor pregnenolone or progesterone to DOC (Table 1, Treatment 1). With

the PABA 4% treatment for 5 or 6 weeks, NADPH supported conversion of pregnenolone was some 20% below control values. In the same preparations, however, with glucose-6-phosphate and NADP, the tissue from the experimental groups showed generally the same or greater levels of conversion of pregnenolone or progesterone compared to controls. After 8 or 12 weeks of 4% PABA administration, adrenal tissue from the experimental animals showed a markedly reduced ability to convert either precursor steroid (Table 1).

A comparison was made of the ability of adrenal homogenate from normal and PABA-fed rats to convert pregnenolone or progesterone into DOC and B (Table 2). In the presence of NADPH to support the conversion, the primary product was DOC, with, as expected, more product arising from progesterone than from pregnenolone. While

TABLE 1

Comparison of the Ability of Adrenal Homogenate from Normal and PABA-Treated Rats to Convert Pregnenolone or Progesterone into Blue Tetrazolium Positive Corticoid Product

	TREATMENT	μg corticoid/10 mg adrenal tissue* Additions with Pregnenolone as Steroid Substrate NADPH 800 μg	G-6-P+ NADP
I	Control	22.5	23.3
	PABA 2%–10 wks.	19.4	25.0
II	Control	27.2	29.9
	PABA 4%–5 wks.	22.6	33.9
III	Control	27.1	25.3
	PABA 4%–6 wks.	23.1	26.9
IV	Control	35.9	52.5
	PABA 4%–8 wks.	24.6	40.8
V	Control	16.1	------
	PABA 4%–12 wks.	10.9	------
	TREATMENT	Additions with Progesterone as Steroid Substrate NADPH 800 μg	G-6-P+ NAD
I	Control	14.9	15.3
	2% PABA–10 wks.	19.2	17.7
II	Control	27.1	25.3
	4% PABA–6 wks.	25.7	28.8
IV	Control	42.2	36.8
	4% PABA–8 wks.	36.0	26.7

* Incubations carried out for 60 minutes in $NAHCO_3$ 20 mM as indicated in text Corticoid determined by blue tetrazolium reaction.

the levels of DOC production were comparable for controls and either experimental group, the DOC values for the PABA-treated groups were slightly but consistently above control. With oxidizable substrates (Table 2) precursor conversion was lower than with NADPH; however, B was the major corticoid product. There was a tendency for PABA-treatment to be associated with a decrease in substrate supported conversion of DOC, produced from pregnenolone or progesterone, into B. The total production of DOC + B from progesterone in the presence of malate was 9.7, 10.5 and 10.7 μg/10 mg tissue for control, 2% PABA and 4% PABA groups respectively. In the same groups, DOC production was 1.6, 1.8 and 6.6 μg/10 mg tissue respectively. Thus the PABA treatment appears to be associated with a reduced *in vitro* conversion of DOC into B by adrenal homogenate.

The influence of PABA treatment on adrenal 11β-hydroxylation was indicated clearly by the lowered adrenal conversion of DOC into B (Table 3). In the presence of NADPH and Ca^{2+} 11mM, B production by adrenal tissue from 2% PABA fed rats was 1 to 4 μg/10 mg tissue lower than control values; with the 4% PABA group there was a 33% reduction in *in vitro* DOC conversion. The most marked changes in 11β-hydroxylation occurred in experimental preparations when *in vitro* steroid conversions were supported by oxidizable intermediates (Table 3). In the tissue from the 2% PABA treated rats there were decreases in substrate-supported conversions of DOC into B, but results were variable. However, with the 4% PABA treated groups there was up to a 50% decrease in B production in the presence of isocitrate, malate or α-ketogluterate.

DISCUSSION

It is generally accepted that hypothyroidism is associated with a decreased metabolism reflected both at the organismic and cellular level. The data reported by McCarthy and Murphree (1960) note that administration of PABA under conditions identical to those used in this study produced a decreased thyroidal 131-I uptake. In terms of the dose of PABA used in this investigation, the 4% treatment was generally associated with a greater reduction in *in vitro* corticoid conversions than was 2% PABA (Tables 1, 2, 3). With the 4% PABA, however, consistent effects on decreasing steroid precursor conversions were noted only after 8 weeks of treatment. Previous study (McCarthy and Murphree, 1960) indicated that 4% PABA was a much more effective agent than was the 2% diet. While some evidence of thyroid

TABLE 2

Conversion of Pregnenolone or Progesterone into DOC + B by Adrenal Homogenates of Normal and Goitrogen-Treated Rats

Steroid Substrate	PREGNENOLONE						PROGESTERONE					
Treatment	Control		2% PABA		4% PABA		Control		2% PABA		4% PABA	
Prduct μg/10 mg	DOC	B	DOC	B	DOC	B	DOC	B	DOC	B	DOC	B
NADPH	10.7	0.7	12.1	0.9	11.1	0.6	18.5	0.9	20,1	0.9	20.9	0.9
Isocitrate	0.0	3.30	0.0	2.9	0.01	2.5	0.8	6.3	1.7	6.0	2.2	5.4
Succinate	0.0	2.9	0.0	3.4	0.6	2.5	1.2	2.1	1.7	2.7	2.1	1.7
Malate	0.0	2.5	0.03	2.3	1.1	2.4	1.6	8.1	1.8	8.7	4.1	6.6

Adrenal tissue was obtained from rats fed PABA for 12 weeks with comparable untreated control rats.
Incubations carried out for 60 min. in NAH_2CO_3 buffer as indicated in text.

TABLE 3

Conversion of DOC into Corticosterone by Adrenal Homogenate in the Presence of NADPH or Metabolic Intermediate*

Addition	μg Corticosterone/10 mg Adrenal Tissue								
	Expt. I			Expt. II			Expt. III		
DOC, 60 μg plus	Control	2% PABA**	4% PABA	Control	2% PABA	4% PABA	Control	2% PABA	4% PABA
NADPH	2.4	1.7	2.9	2.0	2.6	1.9	2.8	2.1	2.0
NADPH + Ca^{2+} 11mM	30.1	29.2	21.1	33.0	31.2	26.6	34.0	30.3	22.2
Isocitrate	13.1	13.9	8.0	34.0	27.9	19.2	.....	.....	.....
α-Ketogluterate	6.0	6.2	4.3	.....	.....	.....	.....	.....	.....
Malate	17.9	10.9	8.9	27.9	27.0	12.6	.....	.....	.....

* Incubations carried out in a total volume of 2.0 ml containing Tris buffer (20 mM) pH 7.4, DOC 60 μg, pyridine nucleotides 800 μg, and Ca^{2+} 11 mM or substrate 10 mM.

** Adrenal tissue obtained from rats fed PABA for 12 weeks.

inhibition was found in rats fed 2% PABA for 2 or 4 weeks, the later workers found adrenal 32-P incorporation and decreased inorganic phosphorus to be unchanged from control levels. Thyroid inhibition, decreased adrenal 32-P and lower adrenal inorganic phosphorus and adrenal atrophy were found in rats with 4% PABA administered for 12 weeks. Thus it is to be expected that marked changes would occur with the 4% experimental diet after 4 or more weeks of feeding.

The general results of this study suggest an impairment in the production and/or utilization of NADPH, in reactions associated with mitochondria in adrenal tissue of PABA-fed rats. These changes contribute to the decreased *in vitro* 11β-hydroxylation in the adrenal homogenate from the treated groups.

Adrenal preparations from rats treated for 5 or 6 weeks were found to show lower conversions of pregnenolone in the presence of NADPH while conversion of the precursor with G-6-P and NADP was unimpaired. The conversion of pregnenolone to progesterone is known to require oxidized pyridine nucleotide, NAD or NADP (Talalay 1957, Gagliano 1967), while the 21-hydroxylation of progesterone into DOC requires NADPH (Ryan and Engle, 1956). Incubation of adrenal homogenate with NADH has been shown to suppress the conversion of pregnenolone to progesterone unless oxidation of the co-factor can be effected (Koritz 1962, 1963). It may be that after 6 weeks of PABA treatment, the reduced *in vitro* conversion of pregnenolone with NADPH (but not with G-6-P + NADP) could reflect reduced oxidation of NADPH to NAD. It is to be noted, however, that in the same tissue preparation (5, 6 weeks PABA, Table 1) progesterone hydroxylation *in vitro* in the presence of NADPH was but slightly affected by the goitrogen diet. It may well have been that alternate mechanisms for NADPH oxidation were reduced in the homogenate prepared from experimental animals. Adrenal tissue from rats given experimental diet for 8 weeks or longer did show reduced steroid conversions in the presence of either NADPH or G-6-P + NAD.

In rat adrenal mitochondria, Peron, Guerra and McCarthy (1965) demonstrated that conversion of DOC into B in the presence of NADPH required high levels of calcium ions (Ca^{2+}). The effect of Ca^{2+} was noted to bring about mitochondrial swelling to facilitate NADPH entry to the hydroxylating enzyme system. The data noted in Table 2 support the suggestion that exogenous NADPH can support the conversion of pregnenolone to progesterone as well as the subsequent 21-hydroxylation of the latter steroid to DOC by tissue

from normal and PABA-treated rats. There was, however, little conversion of DOC into B in the presence of NADPH alone.

The ability of oxidizable substrate to support adrenal mitochondrial 11β-hydroxylation has been demonstrated by Brownie and Grant (1954), Harding *et al.* (1965), Guerra, Peron and McCarthy (1966), Cammer and Estabrook (1967) and many others. These substrates are seen to be oxidized to generate intramitochondrial NADPH to provide reducing equivalent to the cytochrome P_{450} chain to support hydroxylation reactions (Harding, Wong and Nelson 1964, Guerra, Peron, and McCarthy 1966, and Cammer and Estabrook, 1967).

The studies carried out to provide the data for Table 2 lead to the conclusion that adrenal tissue from PABA-treated rats shows a reduced 11β-hydroxylation of DOC. Though the study was carried out with adrenal homogenate so that substrates such as isocitrate were exposed to cytosol enzymes, there was primarily a conversion of pregnenolone or progesterone into B, (controls, Table 2). With the experimental tissue, however, there was generally a decrease in B production with an increase in DOC accumulation. The fact that DOC was formed indicates that the substrates can be oxidized to generate NADPH for the microsomal 21-hydroxylation to form DOC. The microsomal hydroxylation reactions also involve cytochrome P_{450} (Omura *et al.*, 1965). In all cases for 4% PABA, DOC formation with substrates was greater than control tissue. While these data may reflect normal mitochondrial oxidation of substrate there is evidence for decreased 11β-hydroxylation in homogenate from PABA-treated rats.

The results noted in Table 3 support the suggestion that there is a reduction in oxidation of substrates to generate NADPH and/or cofactor utilization to support the conversion of DOC into B in adrenal tisue from PABA rats. It is of interest to note that with Ca^{2+} 11 mM + NADPH, B production by experimental group adrenal tissue was markedly decreased. Studies have been reported on 11β-hydroxylation by rat adrenal mitochondria relating the role of substrate utilization (Guerra, Peron and McCarthy, 1966) and effect of Ca^{2+} on substrate supported DOC hydroxylation (Peron, McCarthy and Guerra, 1966). These reports noted that either isocitrate or Ca^{2+} 11 + NADPH supported maximum conversion of DOC into B. Addition of Ca^{2+} 11 mM to isocitrate (or other substrates) acted to swell mitochondria and inhibit substrate utilization. Under the latter conditions, where substrate use was suppressed by Ca^{2+}, addition of NADPH supported maximum 11β-hydroxylation (Peron, McCarthy and Guerra,

1966). The findings reported here suggest that while substrate oxidation may be decreased, the data on Ca^{2+} + NADPH (Table 3) suggests decreased 11β-hydroxylation may also occur. These findings will have to be confirmed in adrenal mitochondria where effect of PABA treatment on 11β-hydroxylation and cytochrome P_{450} can be evaluated.

While the most marked effect of the experimental treatment was noted on DOC conversion, decreased conversions of pregnenolone and progesterone were also observed in adrenal homogenate from rats on PABA for 12 weeks. Freedland and Murad (1969) studied effects of thyroxine and thyroidectomy on rat adrenal enzymes. These workers found that thyroidectomy had little effect on most enzymes (as isocitrate dehydrogenase) but increased malic enzyme activity. Since the latter study was carried out in the supernatent of a 20,000 $\times$ g sedimentation of adrenal homogenate, the results, therefore, reflect cytosol enzymes rather than mitochondrial enzymes. The data presented here thus lead to the suggestion that the decreased corticoid production is a change reflecting decreased mitochondrial oxidation metabolism involving generation or utilization of NADPH.

These findings of reduced *in vitro* B formation appear to be in opposition to the report of Cortes and Peron (1964) who found that adrenal B and 18-OHDOC secretion in PABA-treated rats was unchanged from control animals. While conversion mechanism could be such that *in vitro* reactions would be lowered, *in vivo* control might bring about conditions for normal synthesis and release. Steinetz and Beach (1963) reported that rats treated with thiouracil show normal response to ACTH *in vivo* but *in vitro* evidence of increased adrenal responsiveness to ACTH was noted. The findings reported here need to be confirmed in adrenal mitochondria and microsomal preparations with additional studies on ACTH responsiveness *in vitro*.

During the course of this study plasma samples of PABA treated rats were subjected to 18-OHDOC determination of Cortes, Peron and Dorfman (1963). In agreement with prior report of McCarthy *et al.* (1959), the levels of Porter-Silber positive components on peripheral plasma of rats given 4% PABA for 12 weeks was 4 to 6 times above normal expected values. At the same time, however, in agreement with the report of Cortes and Peron (1964), adrenal vein secretion of 18-OHDOC in PABA-fed rats was unchanged from control. It may well be that the high level of Porter-Silber positive material in the systemic blood of PABA-treated rats may reflect altered peripheral

metabolism. As yet we have not confirmed that this Porter-Silber position component is in fact 18-OHDOC.

SUMMARY

Adrenal homogenate obtained from rats fed PABA for periods up to 12 weeks evidenced decreased *in vitro* adrenal corticoid conversion. While conversion of pregnenolone or progesterone appeared suppressed following treatment, a more pronounced effect on DOC conversion was noted. The response to added oxidizable substrate or NADPH suggested that co-factor generation and/or utilization was suppressed. Some evidence for decreased use of NADPH for 11β-hydroxylation was found.

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Fine Structure of Nucleoli in Cells of Encysted *Hymenolepis diminuta*

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Studies of the fine structure of 5 day old cysticercoids of *H. diminuta* revealed nucleoli with well-formed lamellae in germinal cells within the body of the encysted worm. The nucleoli were located centrally in the nucleus and appeared not to be attached to the chromosomes, chromatin, or to the nuclear envelope.

A few nucleoli possess nucleolar material attached at their surfaces as lamellae (Fig. 1). The lamellae measure about 60 to 80 mμ in diameter and may arise by splitting from the central bodies of the nucleoli. Occasionally a lamella is branched and appears to communicate with the scattered pars amorpha.

Porter (1954, J. Histochem. Cytochem. 2: 346-355; 1960, Fourth internat. Conference on Electron Microscopy, eds. Bargmann Mollenstedt, Niehrs, Peters, Ruska and Wolpers, Berlin-Göttingen-Heidelberg: Springer) reported lamellae in striated muscle of *Ambystoma* larva, and Beams and Seklon (1968, Zeit. f. Zellforsch. 85: 237-242) found lamellae in oöcytes of centipedes and crayfish. The latter

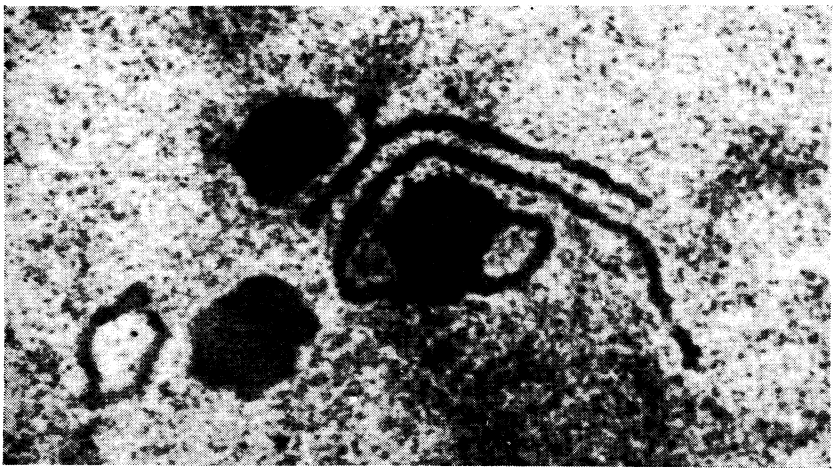

FIG. 1. Nucleoli from germinal cell of 5 day old cysticercoid *Hymenolepis diminuta*.

authors suggested that the lamellae may function to transfer material between nucleoli.

Nucleoli are believed to function as depositories for ribonuclear genetic materials that are reworked and elaborated for cytoplasmic use. They appear to originate in association with certain chromosomes and they are more or less constant in number for given somatic cell types (Moses, 1964, Cytology and Cell Physiology, ed. by Bourne, 3 ed. Academic Press, New York).

Kaulenas, Foor and Fairbairn (1969 Science 163: 1201-1202) reported the presence of nucleoli in 4-celled *Ascaris* embryos that were associated with the synthesis of rRNA. These authors showed an increase in the numbers of nucleoli and the nucleoli exhibited lamellae.

The above evidence indicates that the presence of lamellae, like the increased numbers of nucleoli, are more related to increased RNA production than to transfering of materials between nucleoli.

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