Effects of Aminoglutethimide on Corticosteroids in Adrenal Vein Plasma of the Rat

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Abstract

Aminoglutethimide phosphate (AGP) was given s.c. to male albino rats (Charles-River strain) in 50 mg/Kg body weight doses, one-half hour before withdrawal of blood. Following heparinization, the renal vein was ligated medial and lateral to the confluence of the adrenal vein with the renal vein. Adrenal vein blood was then withdrawn and centrifuged until 2.5 or 3.0 ml of plasma was obtained. By studying the chromatographic plates, it became obvious that AGP had, in some manner, altered the corticosteroid production of the adrenal cortex. A spot, corresponding with the cortisone standard, and tentively designated as such, showed up in large quantity in all of the treated samples, but was not seen in any of the control samples. This seems to indicate that AGP is inhibiting, to a degree, 11-hydroxylation, while inducing 17-hydroxylation.

Aminoglutethimide phosphate,[2] in doses of 250-500 mg three times a day, is moderately effective in controlling nuero-muscular seizures (1). Pittman and Brown (7), working with rats, showed that 250 or 500 mg/Kg caused an increase in thyroid and ovarian weight, as well as an increase in adrenal weight. Dexter *et al.* (3) have reported preliminary evidence that 30 mg of aminoglutethimide per rat modifies steroid synthesis at a step prior to Δ^5-pregnenolone.

Thin-layer chromatography has been shown by several workers (2, 8, 10) to be an effective method for the separation of a mixture of corticosteroids. Since these molecules are so closely related, often differing by only a hydroxyl or keto grouping, different solvent systems can be employed to obtain the best separation. Also, the separations, using thin-layer chromatography, are noticeably sharper than those obtained by paper or column chromatography. Another advantage of thin-layer chromatography is that the sample needs only to contain a few micrograms in order to be seen on a plate, whereas in paper or column chromatography, larger samples are needed to produce spotting.

The problem in the present study was to investigate possible changes in types of corticosteroids in adrenal vein plasma after the administration of aminoglutethimide phosphate. Fluctuations in adrenal vein corticosteroids should reflect changes in release and presumably production of corticosteroids by the adrenal cortex. Thin-layer chromatography was used to separate the corticoid and changes in steroid types were determined by simultaneous comparisons of standards, control samples, and samples from the treated animals.

Materials and Methods

Male albino rats (Charles-River strain) weighing between 350 and 400 grams were used. Treated animals were injected subcutaneously

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with 50 mg/Kg of aminogluetethimide phosphate (AGP). Adrenal vein blood was obtained during a ten minute interval starting about one-half hour after the injection of the drug. This interval was chosen since Eversole and Thompson (4) found that subcutaneous injections induced muscular ataxia in 15-30 minutes after injection, thus indicating rapid absorption. The animals were anesthetized with sodium pentobarbital intraperitoneally and then placed under ether until blood withdrawal was completed. A laparotomy was performed to expose the left kidney and renal vein, the left adrenal gland and its vein from the gland to its point of entry into the renal vein, and the left spermatic vein. The spermatic vein was ligated. A ligature was placed around the renal vein between its confluence with the inferior vena cava and the adrenal vein; a second ligature was placed around the renal vein between the adrenal vein and the hilus of the kidney. Before these were drawn tight, the animal was heparinized, heparin sodium being injected into a branch of the superior mesenteric vein, and then the vein being occluded with a hemostat. The ligatures were then drawn tight, taking precautions against obstructing or constricting the adrenal vein at its confluence with the renal vein. Having performed this step, a pocket is thus formed by the renal vein into which adrenal vein blood will flow. A #23 one-quarter inch needle was inserted into the renal "pocket" and blood was withdrawn over a ten minute period (6.0-8.0 ml of blood collected from 2 or 3 rats). The plasma was separated by centrifuging the collected blood and 2.5 or 3.0 ml of plasma was extracted. The plasma was placed in a graduated centrifuge tube and dichloromethane, 2.5 times the volume of plasma, was added. This was shaken for one minute and then centrifuged. The dichloromethane fraction, the lower portion, was then withdrawn and placed in a beaker. The material remaining in the centrifuge tube was then treated with a second portion of dichloromethane, 2.5 times the original volume of plasma. The two dichloromethane fractions were combined in the beaker, placed in a desiccator, and allowed to evaporate. The sides of the beaker were washed with 1 ml of dichloromethane and the extract was evaporated to dryness once more. The plasma residues, prior to plating, were taken up in 0.5 ml of methanol.

A rectangular chromatography tank was prepared by placing a strip of filter paper around the inside of the tank and adding the solvent (dichloromethane:methanol:water, in the ratio 225:15:1 ml) twelve hours prior to the running of the plate. The plates used were Merck pre-coated analytical grade coated with Silica Gel-F_{254}, containing an inorganic fluorescent indicator. They were placed in a drying oven at 120°C, one hour before they were to be used. Extracted plasma samples and standards (1 μgm/μl in methanol) were applied 2.0 cm from the lower margin of the plate with a 0.01 ml micro pipet, and then the spotted plates were placed in the drying oven for 30 seconds at 60°C to drive off methanol. Each plate was placed in a metal rack, which held it upright, and this assembly was transferred to the chromatography tank and allowed to run 16 cm (30-40 min). After the solvent migration, the plate was taken from the tank and viewed under UV-light

(wavelength 2540 Å), the steroids showed up as dark spots, because they blocked the fluorescent indicator.

Since humidity and temperature varied slightly from day to day, standard samples were run on the chromatographic plate with the treated and control samples.

Results

In viewing the chromatograms it became obvious that corticosteroid production in the rat was definitely being altered in some manner by AGP treatment.

Table 1 shows a numerical representation of the results, in which the density of the spots was approximated on the basis of ++++ being the highest concentration of the substance and + being a spot which was barely visible. Distance migrated referred to the distance the spot moved from its point of application onto the plate to the point it ended up after the run was completed. The R_f value was cal-

TABLE 1

Effects of AGP on Adrenal Vein Steroids

Control Samples			Experimental Samples		
Chromatogram No. 1, 2.5 ml Plasma					
Density Value	Distance Migrated (cm)	R_f Value	Density Value	Distance Migrated (cm)	R_f Value
+++	14.6	.91	+++	14.6	.91
+	11.7	.73	+	11.5	.72
+	10.5	.66	+	10.7	.67
+++	8.6	.54	+++	8.8	.55
+	5.8	.36			
			++	5.1	.32
		Standards:			
		Cortisone		5.2	.32
		Corticosterone		7.3	.46
		Hydrocortisone		3.2	.20
		Aldosterone		4.6	.29
Chromatogram No. 2, 2.5 ml Plasma					
+	12.6	.79	++	12.5	.78
+	11.0	.69			
+	8.5	.53	+	8.2	.51
			+	6.7	.42
+	6.1	.38	++++	6.1	.38
+	4.6	.29			
			++	3.9	.24
++	2.3	.14			
		Standards:			
		Cortisone		3.9	.24
		Corticosterone		5.2	.32
		Hydrocortisone		2.5	.16
		Aldosterone		3.6	.22

Chromatogram No. 3, 3.0 ml Plasma

Density Value	Distance Migrated (cm)	R_f Value	Density Value	Distance Migrated (cm)	R_f Value
++++	11.5	.72	++++	11.6	.73
+	10.4	.65			
+	8.2	.51	+	8.1	.50
			++	6.8	.42
+++	6.4	.40			
			+++	5.8	.36
++	4.8	.30			
			++++	4.2	.26
		Standards:			
		Cortisone		3.9	.24
		Corticosterone		5.2	.32
		Hydrocortisone		2.5	.16
		Aldosterone		3.4	.21

Chromatogram No. 4, 3.0 ml Plasma

++++	12.1	.76	++++	12.2	.76
+	8.7	.54	+	8.6	.54
+	7.9	.49	+	7.8	.49
			++	6.4	.40
+++	5.7	.36			
			+++	5.0	.31
++	4.3	.27			
			++++	3.8	.24
		Standards:			
		Cortisone		3.9	.24
		Corticosterone		4.9	.31
		Hydrocortisone		2.5	.16
		Aldosetrone		3.5	.22

culated as the distance the spot migrated divided by the distance the solvent system migrated (the distance for the solvent system migration was standardized to 16 cm). Since the standards were always applied in a concentration of 1 μgm/μl, no density value for them was needed.

As shown in Table 1, the most obvious result was the appearance of a spot in the region corresponding with the cortisone standard in the treated animals. This spot did not appear in any of the samples from untreated animals, but was present in a high concentration in all samples from the treated animals. An excerpt from Table 1 shows this very close correlation:

CORTISONE STANDARD				TREATED ANIMALS		
Plate Number	Distance Migrated (cm)	R_f Value	Amount of Plasma in Sample (ml)	Density Value	Distance Migrated (cm)	R_f Value
1	5.2	.32	2.5	++	5.1	.32
2	3.9	.24	2.5	++	3.9	.24
3	3.9	.24	3.0	++++	4.2	.26
4	3.9	.24	3.0	++++	3.8	.24

None of the plasma-extract spots had R_f values like those of aldosterone, but a spot with an R_f value similar to hydrocortisone was seen in the control sample on chromatogram no. 2. The average R_f value for corticosterone on the four plates was 0.35. All control and experimental samples exhibited spots with R_f values clustered near ($\pm$ 10%) the corticosterone average, but in only one case (chromatogram 4, experimental sample) were the R_f values identical.

There were some difference in the chromatograms obtained from 2.5 and 3.0 ml experimental plasma samples. The most significant difference related to this study was that the density of the spots associated with cortisone was considerably greater in the more concentrated sample (3.0 ml plasma).

When AGP was run on a plate, it had an R_f value higher than corticosterone (AGP = 0.39; corticosterone = 0.32). It was also found that AGP was not miscible with dichloromethane.

Discussion

In these studies it was demonstrated that AGP was affecting alteration of adrenal blood corticosteroids.

The most obvious change was the appearance of a spot corresponding with the cortisone standard in all of the treated animals; this spot was not present in any of the control animals. This new substance was being produced in high concentrations. In a personal communication, Dr. Sheppard of the CIBA Corporation stated that it was difficult for him to believe that cortisone was being secreted by the rat adrenal cortex. In his own experimentation he found that 18-hydroxy-11-deoxycorticosterone (18-OH DOC) will have an R_f value similar to that for cortisone. The solvent system which he used was dichloromethane, containing 5% absolute alcohol and saturated with a volume of water equal to 5% of the total volume. This solvent system is similar to the one used in this experiment, except that Dr. Sheppard used about ten times as much water, which, being highly polar, might account for the fact that his R_f values for cortisone and 18-OH DOC were similar. Quesenberry and Ungar (8) using a solvent system of dichloromethane:methanol:water:150:9:0.5, obtained R_f values for cortisone and 18-OH DOC, of .48 and .82 respectively. Therefore, the possibility exists that cortisone was actually being formed following administration of AGP.

Studies on the rat adrenal gland have revealed that although the gland does secrete corticosterone, the enzymes for synthesis of 17-hydroxycorticoids are present. In the rat, the distribution of enzymes appear to be such that hydroxylation of carbon-11 (is favored and that this reaction reduces the hydroxylation of carbon-17), so that the former reaction precludes the latter (11). In an experiment on dogs in 1959 by Jenkins, Meaklin, and Nelson (5), it was shown that administration of large doses of metyrapone (SU 4885), an adrenal inhibitor, resulted in a fall in total steroid production. However, with a smaller dosage (75 mg/Kg) there was no reduction in total 17-hydroxycorti-

coids, but there was an inhibition of hydrocortisone production with concomitant secretion of large quantities of 11-deoxyhydrocortisone, indicating that the adrenal 11-hydroxylating enzyme system was especially sensitive to the effects of this drug. Therefore, it seems possible that AGP could be acting by inhibiting 11-hydroxylating mechanisms and thus permitting ketone formation at C-11 and hydroxylation at C-17. Such a process would favor the production and release of cortisone.

Dr. Sheppard (personal communication) also mentioned that the distinctive spots obtained with an R_f of .55 on Plate 1, .38 on Plate 2, .36 on Plate 3, and .31 on Plate 4, could very possibly be AGP. When AGP was run as a separate sample, its R_f value was slightly higher that corticosterone. But when AGP was mixed and centrifuged with dichloromethane, they were not miscible, and it seems that in the plasma, the AGP would have been separated out with other material non-miscible with dichloromethane. The density of these spots showed a substance in high concentration, and it is unlikely that any residual carry over of AGP would have been this concentrated. Further confirmation of the probable absence of AGP in the samples was gathered from the results obtained with extract obtained from 2.5 ml of control plasma; here there was a substance that migrated at the same rate as the four spots mentioned above, yet the plasma extract was obtained from rats that had not been injected with AGP.

One puzzling problem was the apparent lack of conclusive evidence that corticosterone was present, except in one sample. In the rat the steroid highest in concentration (8-33 ugm/100ml peripheral blood) has been reported to be corticosterone (11). The operative procedures used in this experiment required the use of ether and the performance of a laparotomy. Ether has been shown to induce the production of ACTH, and a stress will induce an "alarm reaction," the initial phase of which is the "phase of shock," and is characterized by changes which are similar to those seen in acute adrenal insufficiency (6). Since blood was not withdrawn for fifteen minutes following laparotomy and twenty minutes following administration of ether, the corticosterone in both the control and treated animals, might have been depleted or in low quantity when blood was withdrawn. Another possibility is that corticosterone in the extract was attached to a carrier which slightly impeded its migration, thus accounting for the plasma sample spots clustered near the corticosterone standard.

Hydrocortisone and aldosterone are both released by the rat adrenal in very minute amounts (9), therefore, it is not surprising that neither of these were found on the plates. It would be worthwhile to take a larger blood sample, and in doing this attempt to obtain plasma extracts which show the presence of these and other steroids. Some of the substances were undoubtedly lost during the separation process and by using larger volumes of plasma there would be more assurance that the concentration of corticoids in the extract would be higher and thus show more distinctly as dense spots.

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