Aminoglutethimide Inhibition of Adrenal Desmolase Activity* (32877)

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Inhibition of adrenal corticosteroid biosynthesis by aminoglutethimide (Elipten-Ciba) has been well documented (1–5). Dexter *et al.* demonstrated a block in steroid production at a step prior to Δ-5-pregnenolone in adrenal slices incubated with aminoglutethimide (6). Kahnt and Neher, after studying numerous glutarimides, including glutethimide (Doriden-Ciba), postulated that the inhibitory effect was due to a block in 20-hydroxylation of the parent compound cholesterol (7). The present study was undertaken to define the site and mechanism of action of aminoglutethimide further, particularly with respect to its influence on the submitochondrial cholesterol side-chain desmolase complex extracted from acetone powder of adrenals (8).

Materials and Methods. Assays for cholesterol side-chain cleaving desmolase system were performed according to the method of Kimura *et al.* (9). Cholesterol-26-^{14}C or tritium labeled 20-hydroxycholesterol, suspended with the aid of Tween 80, were used as substrate. Acetone powder extracts of rat or human adrenal tissue were used as the enzyme source. The human material was obtained at postmortem and frozen within 6 hours after death. Aminoglutethimide and glutethimide were suspended with Tween 80 and substrate for inhibition studies in this system. Details of the incubation mixture have been described previously (1).

Following incubation, the samples were heated to dryness at 135°C to remove the volatile labeled side-chain cleaved from cholesterol. Since both substrates were labeled in the side-chain, the decrease in counts per minute during incubation was used as a measure of enzyme activity.

Side-chain labeled 20-hydroxycholesterol was synthesized by a modification of the method of Petrow and Stuart-Webb (10). The 1-bromo-4-methyl-pentane-^{3}H (New England Nuclear Corp.) was prepared by tritium gas exchange and had a specific activity of approximately 200 μC/mg. A 660 mg sample of this material was mixed with 96 mg of magnesium turnings. This radioactive magnesium salt was then allowed to react with 776 mg of silated pregnenolone. The desired product was isolated chromatographically and purified by recrystallization. Its infrared spectrum showed general resemblance to that of cholesterol but with two characteristic hydroxy groups. The specific activity of the final product was 1.5×10^7 cpm/mg.

Aminoglutethimide was determined according to the method of Douglas and Nicholls (11), in which the compound forms a colored product with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). The sensitivity of this method allows accurate determination of 0–15 μg/ml. Glutethimide does not form a colored Schiffs base with this reagent, which distinguished between the two compounds.

Glucose 6-phosphate dehydrogenase activity was measured spectrophotometrically through TPNH production (12).

Thirty 6 week old Sprague-Dawley rats were divided into three groups: 10 animals received daily injections of aminoglutethimide (400–500 mg/kg intraperitoneally); 10 received glutethimide in a daily dosage gradually increasing from 50 to 100 mg/kg intraperitoneally. (Higher doses had marked depressant effects and caused death or excessive sleep, thus interfering with food consumption and growth). The remainder of the animals served as controls and received daily intraperitoneal injections of saline. After 10 days all animals were sacrificed, one adrenal was fixed in formalin and the other was rapidly frozen for enzyme analysis.

Results. Figure 1 shows the inhibitory effect of increasing concentrations of aminoglutethimide and glutethimide on the enzymatic conversion of cholesterol. It will be noted that aminoglutethimide was at least 100 times as potent as glutethimide. The rela-

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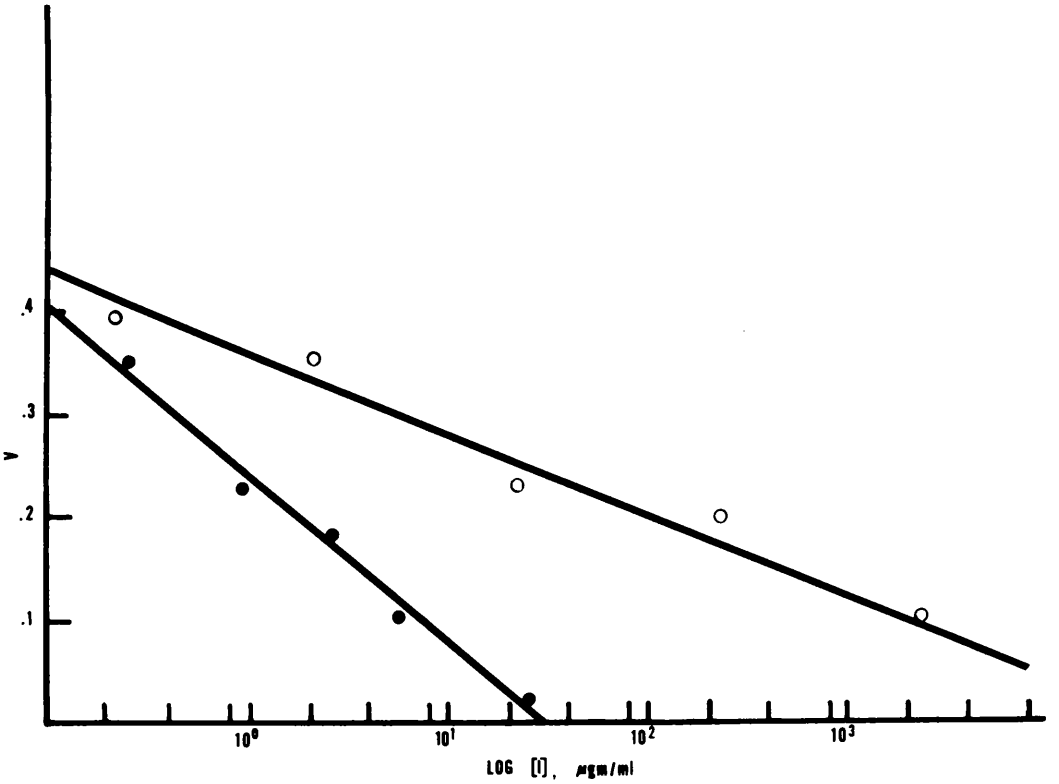

FIG. 1. Effect of aminoglutethimide (●), and glutethimide (○) on the velocity of cholesterol conversion. V = 10—^{9} moles/hour per mg.

tive potency of the two drugs suggested the possibility that the weak inhibitory action of glutethimide may have been due to contamination with aminoglutethimide. However, no aminoglutethimide could be found in 60 mg of glutethimide, with a method of such sensitivity that contamination with 0.1% aminoglutethimide would have given an absorbance reading of 0.125.

Preincubation of whole tissue homogenates with glutethimide or aminoglutethimide for 15–60 min, followed by acetone powder preparation removed essentially all inhibition (the inhibitor compounds were removed with the lipid extraction). This was interpreted as evidence against alteration of the enzyme molecule by these compounds. The possibility that aminoglutethimide was deaminated by the tissue during preincubation and was thus converted to its less potent analogue was excluded by the fact that, following incubation, 95–98% of the aminoglutethimide added to the homogenate could be extracted with dichloromethane.

While control reactions proceeded at a linear rate for 30 min, inhibited incubation mixtures containing 10^{-6} *M* cholesterol were depressed for the first 15 min, after which the velocity increased, reaching that of control mixtures. To explore the possibility that aminoglutethimide might have been irreversibly altered by the enzyme complex and thus its relative concentration effectively decreased during initial reaction times, the drug was preincubated with soluble enzyme for 30–60 min, with or without the TPNH generating system. This aminoglutethimide–enzyme mixture did not inhibit cholesterol conversion. There was no difference between samples preincubated in the presence or absence of TPNH (Table I). Although with the colorimetric determination employed, about 75% of this preincubated aminoglutethimide could be recovered after completion of these reac-

TABLE I. Effect of Preincubation of Enzyme with Inhibitor.[a]

Time (min)	Counts per min[b]			
	Control	Inhibitor added	Preincubated with TPNH	Preincubated without TPNH
0	2935	3044	3101	3058
10	2568	3108	2778	2522
20	2238	3042	2359	2357
30	1858	2669	1925	2052
50	1225	2393	1657	1399

[a] $[S] = 1 \times 10^{-6} M$; $[I] = 15.2 \times 10^{-6} M$.

[b] The decrease in cpm during incubation is a measure of enzyme activity (see "Materials and Methods").

TABLE II. Effect of Aminoglutethimide on Cleavage of 20-OH-Cholesterol.[a]

Time (min)	Counts per min[b]			
	Control	$[I] = 1 \times 10^{-5}$	$[I] = 2 \times 10^{-5}$	$[I] = 5 \times 10^{-5}$
0	2520	2880	2475	2670
5	2408	2551	2190	2551
10	2250	2423	2003	2205
20	1998	1915	1710	2010
30	1523	1710	1575	1755

[a] $[S] = 5 \times 10^{-6} M$.

[b] The decrease in cpm during incubation is a measure of enzyme activity (see "Materials and Methods").

tions, it was recognized that the phenylamine group could continue to complex with Ehrlich's reagent despite any alterations which might have occurred in the ethyl or glutarimide portions of the molecule.

Since the assay for desmolase activity depends on regeneration of TPNH by glucose 6-phosphate dehydrogenase, it was necessary to assess the effect of aminoglutethimide on this enzyme. It was found that up to 2×10^{-4} M aminoglutethimide, which is 25 times the concentration necessary for substantial *in vitro* inhibition of cholesterol conversion, had no effect on glucose 6-phosphate dehydrogenase.

When 20-hydroxycholesterol was used as substrate under similar incubation conditions, up to 76 μM concentration for aminoglutethimide had no effect on its conversion to pregnenolone. The results are seen in Table II; conversion of substrate to product was followed by measuring decrease in radioactivity with time. These results established the block at the initial hydroxylation of cholesterol.

Attempts to overcome the *in vitro* inhibition of cholesterol conversion by aminoglutethimide were performed by increasing the total amount of substrate. It was recognized that, since substrate and enzyme were of a particulate nature and since the former was unevenly suspended in the nonionic detergent, the availability of the substrate for enzyme binding was probably not constant or uniform and that kinetic studies would be difficult. Nevertheless, the inhibitory action was reversed by increasing the substrate concentration and a competitive inhibition was demonstrated, as shown in Fig. 2. Under the conditions used in these experiments, V_{max} was 8×10^{-9} moles per hour and the apparent K_m was 10^{-5} M. The apparent K_m rose with increasing concentrations of inhibitor. From the Dixon plot shown in Fig. 3, the K_i was calculated to be 3.5 μM.

Discussion. The results of *in vitro* studies

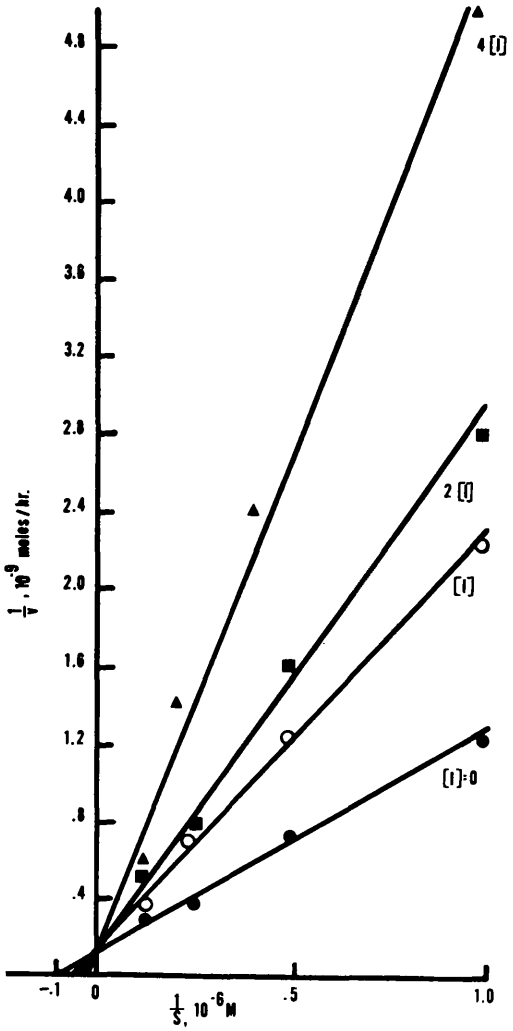

FIG. 2. Double-reciprocal plot demonstrating competitive inhibition. [I] = 3.8 × 10—⁶ M.

confirmed the previous findings that aminoglutethimide blocks the conversion of cholesterol to pregnenolone by adrenal cortical tissue and suggest that this block is achieved through a competitive inhibition of the mitochondrial desmolase side-chain splitting enzyme. This inhibition is achieved through a block in 20-hydroxylation of cholesterol; aminoglutethimide, in similar concentrations, apparently exerts no effect on cleavage of the six-carbon side chain.

The adrenals of the aminoglutethimide treated animals showed the characteristic histologic changes previously described (diffuse hypertrophy, marked increase in cholesterol, and loss of cortical zonation) (1), whereas the glutethimide treated group appeared essentially normal. There was no significant difference in the extractable desmolase activity between pooled adrenals of control, aminoglutethimide, and glutethimide treated rats. These results established that these drugs do not interfere with the synthesis of the enzyme complex.

Since there is little structural similarity between the glutarimide inhibitors and the normal substrate cholesterol, and since the difference between the parent compound and the intermediate 20-hydroxycholesterol resides only in a tertiary hydroxy group, it is difficult to explain the site and mechanism of action. Perhaps the nonpolar inhibitor molecule has some stereospecific similarity, probably residing in the ethyl glutarimide portion, with the hydrophobic portion of the cholesterol molecule which is normally bound by the 20-hydroxyoxygenase. The presence of an amino group enhances the fit between enzyme and inhibitor, through electrical interaction or salt formation and may account for the difference in potency between aminoglutethimide and glutethimide. Aminoglutethimide itself might undergo conversion to a product which then renders it inactive as an inhibitor.

Aminoglutethimide has been employed effectively as a therapeutic agent and deserves

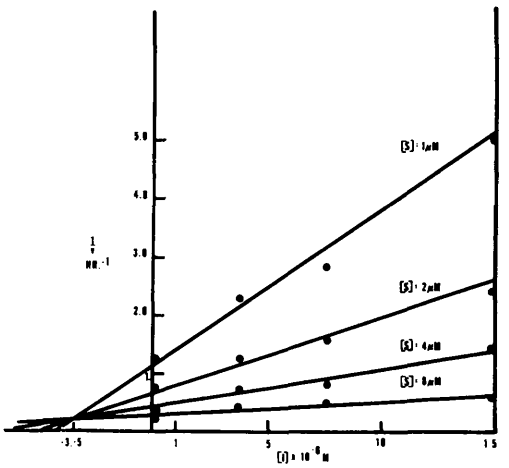

FIG. 3. Dixon plot for four substrate concentrations using aminoglutethimide as inhibitor [I].

further clinical trials (1,14). It is the most promising agent available for situations in which a decrease in corticosteroid production is desirable. Its *in vitro* action affords a unique opportunity for qualitative and quantitative study of the effect of ACTH on steroidogenesis, since the tropic hormone is believed to enhance steroidogenesis through its effect on 20-hydroxylation of cholesterol (15–20). Although glutethimide is less than 1% as potent as aminoglutethimide, its inhibitory action must be reckoned with in cases of attempted suicide with large doses of the drug.

Summary. Aminoglutethimide was found to be a competitive inhibitor of the enzymatic conversion of cholesterol to pregnenolone by the adrenals. This effect is due wholly or in part to an inhibition of the 20-hydroxylation of cholesterol.

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Histochemical Investigation of Hepatic Adenosine Triphosphatase and Glucose-6-phosphatase Activity in Hemorrhagic Shock* (32878)

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The profound effects of hemorrhagic shock upon carbohydrate metabolism and upon hepatic glycogenolysis have been repeatedly documented (1,2). The initial response to hemorrhage is hyperglycemia with an accompanying and progressive increase in the blood levels of lactic and pyruvic acids. In termi-

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