Mechanism of Development of Tolerance to B-Diethylaminoethyl Diphenylpropylacetate (SKF 525-A) in the Rat. (28608)

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SKF 525-A and related compounds are potent inhibitors of drug metabolism whose activity is reflected in an increased duration of pharmacological response in the pretreated animal. Cook *et al*(1) reported that a tolerance to the barbiturate prolonging effects of SKF 525-A could be produced in the rat. Rumke and Bout(2) showed that the action of SKF 525-A is diphasic, the inhibition of drug metabolism being followed by a stimulatory phase. This has been confirmed by Kato *et al*(3) and by Serrone and Fujimoto (4). The mechanism(s) through which SKF 525-A inhibits microsomal drug metabolism is unknown.

Compounds having the ability to stimulate drug metabolism such as phenobarbital, chlorcyclizine and 3-methylcholanthrene caused a marked increase in liver protein (enzyme) synthesis coincident with a strong stimulation of ascorbic acid biosynthesis(5). Scorbutic animals are known to have a decreased ability to metabolize drugs(6,7).

In this report are presented results of studies concerned with the action of SKF 525-A on ascorbic acid biosynthesis, and studies on the mechanism of tolerance to SKF 525-A in the rat. The effects of prolonged dosing with SKF 525-A on ascorbic acid biosynthesis in the rat were studied to determine if impairment of such synthesis could be a contributing factor in the mechanism of action of the inhibitor. Tolerance studies were concerned with the effects of prolonged dosing with the inhibitor on the *in vitro* and *in vivo* metabolism of hexobarbital in the rat, as well as with effects on liver protein synthesis.

Methods. Male, Holtzman rats weighing 120-160 g were maintained on an ascorbic acid deficient diet (Nutritional Biochemicals Co.) each animal being allowed 8 g of food daily with free access to water. They were housed individually in suspension type cages, or in metabolism cages in experiments requiring urine collections.

In vitro metabolism was studied using rat liver slices suspended in Krebs-Ringer phosphate buffer, pH 7.4. Incubations were carried out for 60 minutes in a Dubnoff incubator shaker using an oxygen atmosphere at 37°C, in the presence of added hexobarbital sodium. Total volume of each incubation sample was 5.0 ml and contained about 350 mg of liver slices. Following incubation, unchanged substrate was determined by the method of Cooper and Brodie(8) and results expressed as micrograms of hexobarbital metabolized per gram of tissue per hour.

The *in vivo* rate of metabolism of hexobarbital was inferred from measurement of sleep time. Animals were dosed with 100 mg/kg of the barbiturate and the time between loss and return of the righting reflex measured. Ascorbic acid determinations in urine and tissue samples were made by the dinitrophenylhydrazine procedure of Roe and Kuether(9). No attempt was made to differentiate between free and total ascorbic acid. Liver protein determinations were carried out by the colorimetric method of Lowry *et al*(10). All drug administrations were made by the intraperitoneal route.

Effect of Prolonged Dosing with SKF 525-A on Urinary Excretion and Tissue Levels of Ascorbic Acid. A group of 10 rats received 2 ml/kg of normal saline solution daily for 5 days. Twenty-four hour urine samples were collected and assayed for ascorbic acid. This is designated as the control period. Following the last urine collection the group was divided so that one-half of the animals received a 50 mg/kg dose of SKF 525-A, the remainder receiving normal saline. Thereafter, the respective groups received 25 mg/kg of SKF 525-A or normal saline twice daily through the twelfth day. Twenty-four hour urine

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samples were collected and assayed for ascorbic acid. Following the final urine collection, all animals were sacrificed, and liver, brain, spleen and adrenals were removed and frozen for subsequent ascorbic acid analysis. Liver weights were recorded at time of sacrifice.

Effects of Prolonged Administration of SKF 525-A or SKF 525-A Plus DL-Ethionine on Hexobarbital Sleep Time, In vitro *Hexobarbital Metabolism, Liver Weight and Liver Protein.* Four groups of rats were used to compare the effects of both acute and prolonged treatment with SKF 525-A on hexobarbital sleep time, a control and a treatment group received 25 mg/kg of SKF 525-A daily for 6 days; the appropriate control group received an equivolume injection of normal saline. On the seventh day, the SKF 525-A treated animals received a 50 mg/kg challenging dose of the compound while the control group received saline, 45 minutes prior to 100 mg/kg of hexobarbital. For comparison to repeatedly dosed animals, 2 additional groups of animals received the challenging dose of SKF 525-A or saline 45 minutes prior to hexobarbital. Sleep times were recorded for all groups.

To study the effects of prolonged dosing with SKF 525-A on hexobarbital sleep time in the presence of the amino acid antagonist, DL-ethionine, the above regimen was repeated adding a group of animals which received DL-ethionine in addition to SKF 525-A. Treatment consisted of 50 mg/kg of SKF 525-A alone or with 30 mg/rat of DL-ethionine on the first day, followed by 25 mg/kg of SKF 525-A alone or with 15 mg/rat of DL-ethionine, twice daily, through the fifth day. Preliminary studies indicated that the DL-ethionine regimen alone did not significantly affect hexobarbital sleep time as compared to untreated controls. A saline control group was also studied. On the sixth day these groups were challenged with 50 mg/kg of SKF 525-A forty-five minutes prior to 100 mg/kg of hexobarbital. Control sleep times were obtained using an additional group of animals. Upon awakening, these animals were immediately sacrificed and their livers were removed, weighed, and frozen for subsequent protein analysis.

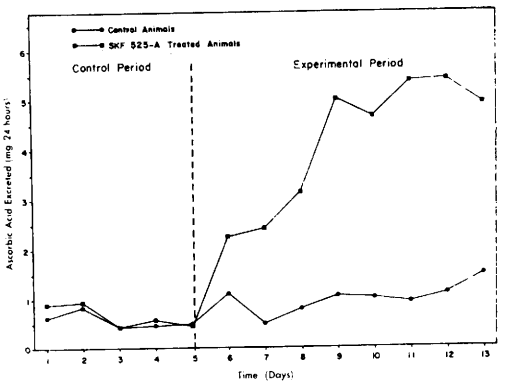

FIG. 1. Urinary ascorbic acid excretion during SKF 525-A treatment.

A similar regimen was followed to study the effects of varying length of pretreatment with SKF 525-A on the *in vitro* metabolism of hexobarbital. The initial dose of SKF 525-A was 50 mg/kg followed by 25 mg/kg twice daily for 3, 5, or 6 days. Dosing was staggered so that all metabolism studies were carried out on the same day. On the day of sacrifice all animals received a 50 mg/kg challenging dose of the compound 45 minutes prior to sacrifice. Control groups received only saline or the 50 mg/kg challenging dose of SKF 525-A were also studied. Metabolism studies were carried out as described.

Results and discussion. Fig. 1 shows the effect of prolonged dosing with SKF 525-A on ascorbic acid excretion in the rat. A marked increase is noted following the first dose, with levels approaching 600% of control values being reached by the fourth day. Stimulation of ascorbic acid excretion is a general property of drugs having the ability to induce increases in microsomal drug metabolism. Such stimulation in the SKF 525-A treated animal appears to be a biochemical correlate in the diphasic action of this compound, and is coincident with development of tolerance to its hexobarbital prolonging effects as discussed below. Analysis for ascorbic acid of liver, brain, spleen and adrenals from SKF 525-A treated animals shows (Table I) that only in the liver (site of synthesis) are there significant differences from controls. It may be concluded that the increased excretion of the vitamin in treated animals represents an increased biosynthesis.

TABLE I. Effect of Prolonged Dosing with SKF 525-A on Tissue Ascorbic Acid and Liver Weight in the Rat.*

	Ascorbic acid, mg % ± S.E.†				Liver wt, g/100 g body wt ± S.E.†
	Liver	Adrenals	Brain	Spleen	
Control	22.9 ± .7	709.6 ± 64.7	28.6 ± .1	65.3 ± 7.4	2.92 ± .02
Treated	28.8 ± 1.3	720.2 ± 37	27.1 ± .6	71.2 ± 5.3	3.95 ± .11
	$p < .01$	$p > .5$	$p > .2$	$p > .5$	$p < .001$

* Data analyzed by student "t" test. Five animals/group. † S.E. = standard error.

It is also noted in Table I that liver weights of the SKF 525-A treated animals are significantly heavier than those from controls. While these studies were in progress, Kato *et al*(3) reported an increased excretion of ascorbic acid in the SKF 525-A treated rat, coincident with a diphasic action of the compound on the metabolism of meprobamate, strychnine and hexobarbital. Their data show that the ability of SKF 525-A to induce increased ascorbic acid excretion is shared by other drug metabolism inhibitors.

The effects of repeated dosing with SKF 525-A on the *in vitro* and *in vivo* metabolism of hexobarbital by the rat are shown in Table II. It is clear from these data that a tolerance develops to the metabolism inhibiting properties of SKF 525-A, which is coincident with a significant increase in liver weight and an increased biosynthesis of ascorbic acid. This would suggest that the mechanism of the tolerance concerns an adaptive increase in the net amount of enzyme present which is responsible for hexobarbital metabolism. A possibility also exists that upon repeated administration the metabolism of SKF 525-A itself is increased, so that the amount of inhibitor available at the cellular level is reduced. This question is being investigated.

Data obtained in studies using DL-ethionine in addition to SKF 525-A in which sleep times, liver weights and liver protein were measured, suggest that the actual mechanism of the tolerance is involved with an increased protein (enzyme) synthesis. It was not possible (Table III) to block completely the secondary stimulation of hexobarbital metabolism in the SKF 525-A treated animals by simultaneous administration of DL-ethionine,

TABLE II. Hexobarbital Sleep Time and *In Vitro* Hexobarbital Metabolism in the SKF 525-A Pretreated Rat.

Hexobarbital sleep time		*In Vitro* metabolism	
Treatment	Sleep time (min)	Treatment‡	Hexobarbital metabolized (μg/g liver/hr)
Prolonged			
(a) 6 days pretreatment with SKF 525-A + 50 mg/kg challenging dose	172.7 ± 17.7*(6)†	(a) Saline control	454.7 ± 32.9*(5)†
(b) 6 days saline control	21.6 ± 19.4 (5)	(b) 50 mg/kg challenging dose only	175.2 ± 32.9 (5)
		(c) One day pretreatment	230.3 ± 32.9 (5)
Acute			
(c) 50 mg/kg challenging dose	404.2 ± 19.4 (5)	(d) Three day pretreatment	221.5 ± 32.9 (5)
(d) Saline control	23.0 ± 19.4 (5)	(e) Five day pretreatment	300.1 ± 32.9 (5)
		(f) Six day pretreatment	244.8 ± 32.9 (5)
(a) *vs* (c) $p < .01$		(e) and (f) *vs* (b) $p < .05$	

* Standard error. Data analyzed by analysis of variance(12).
† No. of animals.
‡ Days pretreated with SKF 525-A. All animals except saline controls received a 50 mg/kg challenging dose of SKF 525-A, 45 min prior to sacrifice.

TABLE III. Blocking Effect of DL-Ethionine on SKF 525-A Induced Increases in Hexobarbital Metabolism, Liver Weight and Liver Protein in the Rat.

Treatment	Sleep time* (min) ± S.E.†	Liver wt (g/100 g body wt)	Liver protein (g/100 g body wt)
(a) Controls—received saline—five days	24.0 ± 30.1 (10)‡	3.37 ±.1 (10)	.73 ±.029 (10)
(b) SKF 525-A pretreated—five days + challenging dose	155.9 ± 30.8 (9)	4.25 ±.11 (9)	.82 ±.031 (9)
(c) SKF 525-A—challenging dose only	317.4 ± 33.7 (8)	3.28 ±.12 (8)	—
(d) SKF 525-A + DL-ethionine 6 days + challenging dose	228.1 ± 30.1 (10)	3.87 ±.10 (10)	.66 ±.029 (10)
	(c) *vs* (d) $p > .05$ (c) *vs* (b) $p < .05$ (d) *vs* (b) $p > .05$	(b) *vs* (d) $p > .05$ (d) *vs* (a) $p > .05$ (b) *vs* (a) $p < .05$	(b) *vs* (a) & (d) $p < .05$

* After 100 mg/kg hexobarbital.
† S.E. = standard error. Data analyzed by Neuman-Keuls test(12).
‡ No. of animals.

although a comparison of difference between the 2 treated groups and the control group reveals that a partial block has been affected. This is also inferred from the liver weights. In sleep time studies, the response of animals receiving both SKF 525-A and DL-ethionine was not significantly different from animals receiving only the challenging dose of SKF 525-A, whereas those animals receiving SKF 525-A alone for 5 days had significantly shorter sleep times than aimals receiving only the challenging dose of the compound 45 minutes prior to the barbiturate. Those animals receiving SKF 525-A alone for 5 days had significantly heavier livers than animals receiving DL-ethionine in addition to SKF 525-A, both groups being significantly heavier than controls. Measurement of liver protein in animals receiving SKF 525-A with or without DL-ethionine when compared to controls gives a clearer indication of the blocking effect of DL-ethionine on induction of protein synthesis by SKF 525-A. This is undoubtedly the most valid consideration, since it has been shown by Dick *et al*(11) that prolonged administration of SKF 525-A in the dog can cause biochemical alterations in liver not related to protein synthesis, and which would not be apparent in measurements of gross liver weights.

Summary. Data presented here substantiate the early finding of Cook *et al*(1) that a tolerance develops to the hexobarbital prolonging effects of SKF 525-A in the rat. It is further shown that the biochemical correlates of this tolerance are an increased biosynthesis of ascorbic acid and an increased liver protein (enzyme) synthesis, which can be blocked by simultaneous administration of DL-ethionine. These observations are consistent with the action of several agents having the ability to stimulate drug metabolism (5). The mechanism of the tolerance, then, may be attributed to an induced metabolic alteration or adaptation to prolonged administration of SKF 525-A. Increased biosynthesis of ascorbic acid in animals repeatedly administered SKF 525-A indicates that impairment of ascorbic acid biosynthesis is not a related factor in the mechanism of action of this drug metabolism inhibitor.

This work supported in part by Nat. Inst. Arthritis and Metab. Dis. grant. We gratefully acknowledge the generous donation of SKF 525-A by Smith, Kline and French Laboratories, and of Evipal (hexobarbital) Sodium by Sterling-Winthrop Research Inst.

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Received April 29, 1963. P.S.E.B.M., 1963, v114.

A Relationship Between Ovarian Norepinephrine and Breast Cancer in Humans.* (28609)

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In a recent study(1) on the distribution of norepinephrine and epinephrine in human tissues, it was observed by one of us (A.H.A.) that ovaries contained a relatively large amount of norepinephrine. Because of the postulated implication of this catecholamine in ovarian function(2), this observation was pursued further and it was found that the high levels of ovarian norepinephrine were associated with breast cancer.

Methods. Segments of ovaries were obtained from 38 patients, 12 of whom had breast cancer, at the time of surgery. (Table I). The catecholamine content of fresh tissue (designated as "normal" after gross examination by a pathologist) was determined by the aluminum oxide-trihydroxyindole procedure of Anton and Sayre(1); fluorescence was measured in the Aminco-Bowman Spectrophotofluorometer. Descending paper chromatography of the aluminum oxide extracted ovarian tissue was carried out on Whatman No. 3 using a solvent system consisting of butanol: acetic acid: water (4:1:5). The catecholamines were located by spraying the paper with a 5% aqueous solution of ethylenediamine, heating the paper at 100°C for 5 minutes and then examining the chromatogram under U.V. lite. Patients receiving catecholamines during the operation were excluded from this study.

* This investigation was supported in part by N.I.H. research grants.

Results. There was a markedly significant elevation of norepinephrine in the ovaries of the breast cancer patients (Table I and Fig. 1). *All* of these patients had 0.50 μg or more of norepinephrine/g tissue whereas *90%* of the other patients had less than this amount. That the catecholamine extracted from the ovaries was indeed norepinephrine was established by means of paper chromatography in which the extract was compared to authentic norepinephrine. Only norepinephrine was detected on the chromatogram since the concentration of epinephrine (or other catecholamines) was below the sensitivity of the paper chromatographic procedure. Further verification of the identity of the extract was obtained by demonstrating that its fluorescence characteristics coincided with those of authentic norepinephrine (Fig. 2). Another intriguing finding was that the corpora lutea in these ovaries had negligible amounts of norepinephrine.

Discussion. It must be emphasized that an association between breast cancer and high levels of ovarian norepinephrine was the only correlation detected. Thus, when an attempt was made to relate ovarian norepinephrine levels to the patient's age, race, stage of pregnancy, phase of menstrual cycle, menopause, pathology (other than breast cancer), or degree of stress, *e.g.*, toxemia of pregnancy, no significant patterns or differences emerged (Table I). Also, the therapy, *e.g.*, drugs, ra-