Biochemical Pharmacology, Vol. 16, pp. 1323–1337. Pergamon Press Ltd. 1967. Printed in Great Britain

MULTIPLE ACTION OF 2′-DIETHYLAMINOETHYL 2,2-DIPHENYLPENTANOATE HCl (SKF 525-A) ON RAT LIVER TRYPTOPHAN PYRROLASE *IN VIVO**

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(*Received* 10 *October* 1966; *accepted* 3 *January* 1967)

Abstract—SKF 525-A was observed to have an early stimulatory action *in vivo* on liver tryptophan pyrrolase activity in intact or adrenalectomized, but not hypophysectomized, male adult rats. The stimulatory effect was abolished by co-administration of actinomycin D, puromycin, or cycloheximide in intact animals, suggesting that SKF 525-A was inducing *de novo* formation of apo-tryptophan pyrrolase. Concurrent with stimulation of tryptophan pyrrolase in intact animals by SKF 525-A, there was a significant decrease in adrenal ascorbate. These effects of SKF 525-A on tryptophan pyrrolase or adrenal ascorbate were not seen in hypophysectomized rats. The stimulation of tryptophan pyrrolase activity by SKF 525-A would therefore seem to involve some form of pituitary activation.

When SKF 525-A was co-administered with hydrocortisone, the stimulatory effects of the two agents were merely additive. However, administration of SKF 525-A to adrenalectomized rats receiving repeated injections of hydrocortisone markedly potentiated the inducing action of hydrocortisone. This enhancement of hydrocortisone induction occurred only if SKF 525-A was administered during the early tryptophan pyrrolase induction phase, i.e. prior to development of attenuation of hydrocortisone induction. Such an effect of SKF 525-A was not obtained in hypophysectomized rats undergoing similar treatments. SKF 525-A-like enhancement of hydrocortisone-induced tryptophan pyrrolase increases could also be produced by injection of saline homogenates of rat pituitary glands during the early tryptophan pyrrolase induction phase.

Animals pretreated 12 hr before with SKF 525-A showed a reduced capacity to form new tryptophan pyrrolase after hydrocortisone administration. This late "anti-inductive" action of SKF 525-A was also seen for a second "stimulatory" dose of SKF 525-A but was not present for substrate stimulation of tryptophan pyrrolase (caused by injected tryptophan). These latter data indicate that SKF 525-A may reduce the ability of the liver to produce tryptophan pyrrolase from other than pre-existing mRNA template.

It is concluded that SKF 525-A effects on hepatic tryptophan pyrrolase are multiple, ranging from hydrocortisone-like to actinomycin-like. No simple explanation for such diverse activities can be offered but this should serve as a caution to investigators who regard SKF 525-A as pharmacologically inert (Mannering, *Ann. N.Y. Acad. Sci.*, **123**, 108 (1965); Brodie *et al.*, *Science* **148**, 1547 (1965) or as a specific inhibitor of microsomal enzyme systems in liver.

SKF 525-A has been widely investigated for its inhibition *in vitro* and *in vivo* of a wide variety of drug- and steroid-metabolizing enzymes in liver microsomes.[1–6] Intraperitoneal administration of SKF 525-A to rats in effective doses (1–100 mg/kg)

* Supported by U.S. Public Health Service Grants CA-05648 and 5-T01-GM01308. A preliminary communication of this work has appeared in *Pharmacologist* **8** 2, 195 (1966).

produces inhibition of hepatic drug metabolism detectable *in vitro* or *in vivo* within a matter of minutes. These effects may be maintained with some enzymic pathways for approximately 24 hr. This inhibition can then be followed by a "rebound" stimulation of some drug metabolisms which persists for approximately 48 hr or more.[7-10] SKF 525-A appears rapidly in the liver after i.p. administration, and disappears within a matter of hours,[10] possibly because SKF 525-A is metabolized by microsomal enzymes.[11, 12]

SKF 525-A binds "irreversibly" to hepatic microsomes *in vitro* and *in vivo*[10, 13] and may cause marked disturbances in hepatic parenchymal cell ultrastructure, as evidenced by vesiculation of the membranes of the endoplasmic reticulum and dissociation of membrane-bound ribosomes.[14, 15] In organs that possess a fairly extensively developed "rough" endoplasmic reticulum (e.g. pancreas and liver), there is evidence to suggest that membrane-bound ribosomes (polysomes) perform the major portion of cellular protein synthesis, while isolated ribosomes are relatively inactive except in the presence of added polynucleotide template ("mRNA").[16-22] SKF 525-A has been shown to inhibit microsomal protein synthesis *in vitro*.[23] Such an action might be due to ribosome disaggregation as is apparent from electron micrographs after administration *in vivo*[14, 15] or to physicochemical effects of microsomal binding of this agent. It therefore seemed possible that SKF 525-A might affect hepatic enzyme inductions such as glucocorticoid induction[24] of tryptophan pyrrolase.† The present experiments indicate that such an inhibitory effect with SKF 525-A is obtained on tryptophan pyrrolase induction, but that it is preceded by a paradoxical stimulation of this enzyme which seems to involve induction.[25]

EXPERIMENTAL PROCEDURE

Animals. Male, Sprague–Dawley rats were used in all experiments. These rats were usually obtained from Simonsen Laboratories, Minneapolis, Minn., and were allowed free access to food and tap water at all times. The adrenalectomized animals used in our experiments were so prepared under ether anesthesia and were given 1% NaCl and 5% glucose in their drinking water for 3–4 days postoperatively. Hypophysectomized Sprague–Dawley rats were obtained from Hormone Assay Labs., Chicago, Ill; these animals were rehydrated with 5% glucose added to their drinking water and were used within 24 hr of arrival. Except where otherwise indicated, all rats were in the weight range of 150–300 g.

Assay procedures. All animals were sacrificed between 8 a.m. and 3 p.m.; they were killed by a blow on the head. Livers were removed, rinsed, blotted, and quickly frozen on dry ice. These livers were maintained frozen in a deep freeze until assayed for tryptophan pyrrolase activity. Homogenization of the thawed livers was performed in a Potter–Elvehjem homogenizer with a loosely fitting Teflon pestle. To each gram of liver was added 3 vol. of ice-cold 0·15 M KCl containing 1·0 mM Na_2 EDTA[26] and 2·5 mM NaOH.[27] In studies where codeine demethylation was also measured, the livers were homogenized in 0·15 M KCl immediately after removal from the animal. These homogenates, in KCl only, were divided, and the remaining

† Abbreviations used in this paper are tryptophan pyrrolase, L-tryptophan: O_2 oxidoreductase, EC 1.13.1.12 (formerly 1.11.1.4.); formylase: aryl-formylamine amidohydrolase, EC 3.5.1.9; mRNA, "messenger" ribonucleic acid; RNA polymerase, nucleoside triphosphate: RNA nucleotidyl transferase, EC 2.7.7.6; ACTH, adrenocorticotropin.

constituents (EDTA and NaOH) were added with rehomogenization to that portion of homogenate reserved for subsequent tryptophan pyrrolase assay. After centrifugation of the homogenates at 9000 *g* in the cold room for 20 min the post-mitochondrial supernatant fractions (cell sap containing tryptophan pyrrolase plus the microsomal fraction) were assayed for tryptophan pyrrolase activity or codeine *O*-demethylase where applicable. Two ml of 9000-*g* supernatant, containing material from 500 mg liver, was incubated with shaking in a Dubnoff incubator under oxygen for 60 min at 37°, as described by Knox,[27] and in the presence of added ascorbate (10 mM).[28–30] Two beakers of 4-ml final volume containing added substrate (3·0 mM L-tryptophan), and one beaker without tryptophan (blank), were incubated for each rat. The neutralized, acid-soluble fraction, absorbing maximally at 365 mμ was measured in a Gilford model 3000 spectrophotometer. This acid-soluble supernatant fraction showed a single peak, at 365 mμ, when compared with that from the blank (no tryptophan), indicating complete conversion of the product of the tryptophan pyrrolase reaction, namely *N*-formylkynurenine, to kynurenine (i.e. formylase activity† was in excess). Each unit of enzyme produces 1 μmole kynurenine after 1-hr incubation under the stated conditions. Hematin, prepared from commercial hemin (Nutritional Biochemicals) as described by Knox and Ogata,[29] was added where indicated in a final concentration of 0·5 μM. Feigelson and Greengard[31] have shown that hematin addition in excess of this concentration is less effective. Hematin was not added routinely to our assay flasks, because tryptophan pyrrolase assays were conducted in the presence of the normal complement of microsomes. Furthermore, addition of hematin disclosed little unconjugated enzyme (data presented in Table 2), and routine addition of this component was not felt to be necessary.

Codeine *O*-demethylation was assayed, under conditions described previously, by colorimetric estimation of morphine formation.[10]

Adrenal ascorbate was measured in quickly frozen adrenals by Maickel's adaptation of Sullivan's method.[32]

Significance values were derived by Student's *t* test.

Drugs. All drugs were administered i.p. except where otherwise indicated. The following drugs or chemicals were injected in aqueous solution: SKF 525-A (from Smith Kline & French Labs.), actinomycin D (from Merck & Co.), puromycin dihydrochloride (from Nutritional Biochemicals and as a gift of the Cancer Chemotherapy Screening Center, Bethesda, Md.), cycloheximide (from Nutritional Biochemicals), 5-fluorouracil (from F. Hoffmann-La Roche, Inc.), hydrocortisone 21-phosphate disodium (from Merck & Co.), and hydrocortisone 21-hemisuccinate (from the Upjohn Co.). L-Tryptophan (from Nutritional Biochemicals) was dissolved in a minimal amount of NaOH,[27] and was then injected immediately.

RESULTS

Effect of SKF 525-A and hydrocortisone, alone or co-administered, on rat liver tryptophan pyrrolase and adrenal ascorbic acid

Initial experiments disclosed a 2–3-fold stimulation by SKF 525-A of liver tryptophan pyrrolase activity in intact rats. The maximal response to SKF 525-A occurred in approximately 4 hr (Fig. 1, top). After this stimulation, tryptophan pyrrolase activity returned to, but not below, control levels. Concurrent with the increase in

liver tryptophan pyrrolase activity, SKF 525-A caused a 30 per cent decrease in adrenal ascorbate (Fig. 1, bottom).

The observed decrease in adrenal ascorbate caused by SKF 525-A probably represents an ACTH- or vasopressin-like-mediated release of ascorbic acid rather than a

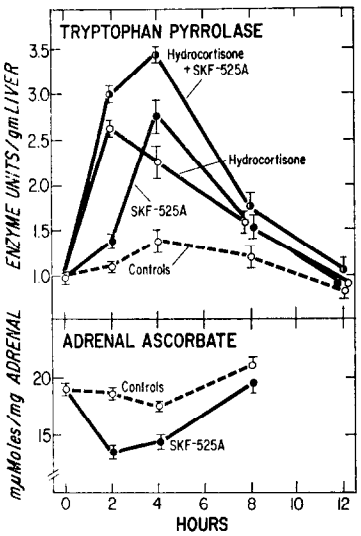

FIG. 1. The early stimulatory effect of SKF 525-A on rat liver tryptophan pyrrolase and adrenal ascorbate release. Top: Time course of stimulation of tryptophan pyrrolase by SKF 525-A [100 μmole (39 mg)/kg i.p.] or hydrocortisone-21 phosphate disodium [10 μmole (5 mg)/kg, i.p.] alone, and simultaneously administered to intact male rats. Control animals were sham injected. Each point represents approximately 11 animals (range: 8–18). Bottom: Time course of adrenal content of ascorbic acid after SKF 525-A (100 μmole/kg, i.p.). Control animals were sham injected. Each point represents approximately eighteen animals (range: 11–35). In both parts of figure vertical bars represent ±S.E.

decrease in adrenal biosynthesis of ascorbate.[9, 33] This suggestion is further supported by our observation that SKF 525-A had no measurable effect on adrenal ascorbate in hypophysectomized rats (Table 1).

Data in Table 1 also show that there was no appreciable stimulation of hepatic tryptophan pyrrolase in hypophysectomized animals treated with SKF 525-A with dosage and time conditions which, as seen in Fig. 1, were effective in intact rats. That this ineffectiveness was not due to lack of absorption of SKF 525-A in hypophysectomized rats is shown by the marked depression of codeine *O*-demethylation by the liver 9000-*g* supernatant fraction from hypophysectomized rats receiving the drug (Table 1).

Of particular significance is the observation that adrenalectomized rats were responsive to SKF 525-A. Heptic tryptophan pyrrolase activity increased approximately 2-fold within 4 hr of administering SKF 525-A to adrenalectomized animals (Table 1).

When SKF 525-A and hydrocortisone were co-administered, it was found that the tryptophan pyrrolase activity was a summation of the effect of either drug given separately (Fig. 1, top). For example, at 4 hr, the net increase in tryptophan pyrrolase

(treated minus controls) following SKF 525-A given alone, was 1·4 units/g liver; the net increase in enzyme activity after hydrocortisone alone was 0·9 units/g liver; and after both drugs co-administered, the net increase in tryptophan pyrrolase was 2·2 units/g liver. These data suggest that SKF 525-A was not affecting tryptophan pyrrolase only by inhibiting the degradation of hydrocortisone, even though SKF 525-A

TABLE 1. TRYPTOPHAN PYRROLASE ACTIVITY, ADRENAL ASCORBATE CONTENT, OR CODEINE DEALKYLATION IN HYPOPHYSECTOMIZED OR ADRENALECTOMIZED RATS RECEIVING SKF 525-A

Exp. no.	Conditions	No. of rats	Adrenal ascorbate (mμmole/mg)	Tryptophan pyrrolase (units/g)	Codeine metabolism (mμmole/g/30 min)
1	Hypophysectomy	8	21·1 ± 6		
	+ SKF 525-A	8	21·2 ± 6		
2	Hypophysectomy	8	21·7 ± 0·4	1·1 ± 0·1	
	+ SKF 525-A	10	19·8 ± 0·4	1·1 ± 0·1	
3	Hypophysectomy	9	18·9 ± 0·5	1·4 ± 0·1	240 ± 15
	+ SKF 525-A	9	18·5 ± 0·9	2·0 ± 0·2	186 ± 15
					P < 0·01
4	Adrenalectomy	8		0·70 ± 0·07	
	+ SKF 525-A	8		1·7 ± 0·2	
				P < 0·01	

Control animals were sham injected. Treated rats received 100 μmole SKF 525-A/kg, i.p. Assays were performed on adrenals or livers 4 hr after treatment. Values represent mean ± S.E. SKF 525-A produced significant effects, on the parameter measured, only in experiments 3 (codeine metabolism) and 4 ($P < 0·01$).

has been reported to inhibit or alter the metabolism of hydrocortisone[34] and other steroids.[6] Furthermore, the time course of stimulation of tryptophan pyrrolase by SKF 525-A lags far behind the rate of passage of SKF 525-A into the liver.[10]

Dose–reponse relationship between stimulation of liver tryptophan pyrrolase and adrenal ascorbate release by SKF 525-A

The apparent need for an intact pituitary in SKF 525-A-induced tryptophan pyrrolase stimulation suggested at least two mechanisms for this enzyme stimulation: (1) SKF 525-A-produced pituitary–adrenal activation and release of corticosterone into the circulation, and (2) "direct" action of an unknown hypophysial component. The latter possibility was suggested when SKF 525-A caused an increase in tryptophan pyrrolase activity in adrenalectomized rats (Table 1).

Since SKF 525-A seemed to produce pituitary–adrenal activation, it was expected that a dose–response analysis of tryptophan pyrrolase stimulation and adrenal ascorbate release in intact rats would disclose similarities in changes in the two parameters studied. It will be seen (Fig. 2) that this was so, and that increases in dosage of SKF 525-A from 100 to 200 μmole/kg produced no further increase in tryptophan pyrrolase or ascorbate depletion.

Studies of possible mechanisms by which SKF 525-A causes increases in tryptophan pyrrolase activity; Effects in vitro *of SKF 525-A, effects on lag phase, effects on apo- vs. holo-enzyme ratios, and evidence for enzyme induction*

Stimulation of tryptophan pyrrolase activity by SKF 525-A could result from several mechanisms, including (1) alteration of amount or availability of the microsomal protoporphyrin component, (2) elimination of the early lag phase in the

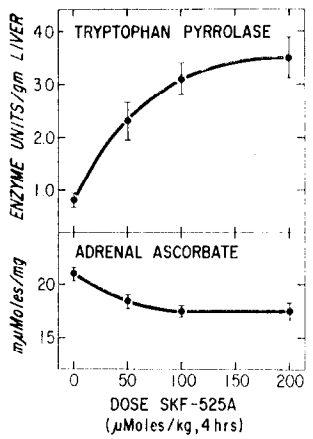

FIG. 2. Dose–response relationship of the SKF 525-A effect on tryptophan pyrrolase and adrenal ascorbate. Top: Effect of graded doses of SKF 525-A on tryptophan pyrrolase activity in intact rats. Zero-dose controls received injections of water (10 ml/kg, i.p.). Bottom: Dose–response relationship between adrenal ascorbate release and dose of SKF 525-A. The same animals were used in assay of tryptophan pyrrolase activity and adrenal ascorbate 4 hr after injection. All doses of SKF 525-A produced statistically significant effects ($P < 0.01$) both on tryptophan pyrrolase activity and adrenal ascorbate levels. In both parts of figure vertical bars represent $\pm$S.E. The values for adrenal ascorbate represent mμmoles/mg adrenal tissue (wet weight).

enzyme activity vs. time curve by activation of the enzyme to its catalytically active (reduced) form, (3) alteration of affinity of the enzyme for its substrate or cofactor, or (4) alteration of the rate of synthesis or decay of the apoenzyme.

Incubation *in vitro* of SKF 525-A with the usual assay mixture (containing 9000-*g* supernatant fractions) at concentrations from 10^{-6} M to 10^{-3} M had no effect on tryptophan pyrrolase activity. This suggests that the "detergent" action of SKF 525-A on liver microsomes[14, 15] was not activating tryptophan pyrrolase by possibly enhancing contact of this enzyme with its microsomal cofactor.

Other experiments were performed with the soluble fraction (105,000-*g* supernatant fraction) from normal vs. SKF 525-A-treated animals. Hematin (0·5 μM) was added to these soluble fractions, and tryptophan pyrrolase activity was measured kinetically by continuous recording of kynurenine formation; i.e. enzyme activity was measured during the linear portion of the activity-vs.-time curve. Under these conditions, 2- to 3-fold increases in tryptophan pyrrolase activity (as compared with untreated animals) were still obtained in liver fractions (cell sap) taken from animals treated 4 hr previously with a dose of 100 μmole SKF 525-A/kg. This indicates that the increase in enzyme activity in animals receiving SKF 525-A was not due simply to shortening or elimination of the tryptophan pyrrolase "lag phase."

Addition of hematin to assay mixtures can disclose the degree to which the apoenzyme is conjugated with its cofactor and thereby provides a means by which two of these possible mechanisms (nos. 1 and 3 given above) may be tested.[35–37] It will be seen from the data in Table 2 that under several conditions, addition of hematin

TABLE 2. EFFECT OF ADDED HEMATIN ON TRYPTOPHAN PYRROLASE ACTIVITY IN LIVER FROM HYPOPHYSECTOMIZED, ADRENALECTOMIZED, OR INTACT RATS TREATED WITH SKF 525-A AND HYDROCORTISONE

All assays were performed on the 9000-*g* fraction as described in Experimental Procedure. Hematin (freshly prepared from commercial hemin) was added *in vitro*, where indicated, in a final concentration of 0·5 μM.[29] Control animals received water (10 ml/kg, i.p.). SKF 525-A was given in a dose of 100 μmole/kg, i.p. Hydrocortisone hemisuccinate was given in a dose of 9·6 μmole (5 mg)/rat (rats weighed 160–175 g). SKF 525-A and hydrocortisone were administered i.p. 4 hr before sacrifice of animals for assay. Values in table are mean ± S.E., obtained from experiments on four animals.

Treatment	Tryptophan pyrrolase (units/g liver) Hematin added		Ratio of enzyme activity + hematin
	No	Yes	− hematin
Hypophysectomized			
Controls	1·2 ± 0·1	1·4 ± 0·1	1·2
SKF 525-A	1·6 ± 0·1	2·0 ± 0·1	1·2
Hydrocortisone	5·2 ± 0·4	6·8 ± 0·4	1·3
SKF 525-A + hydrocortisone	6·2 ± 0·5	7·2 ± 0·2	1·2
Adrenalectomized			
Controls	0·73 ± 0·1	0·83 ± 0·2	1·1
SKF 525-A	1·5 ± 0·1	1·6 ± 0·2	1·1
Hydrocortisone	3·6 ± 0·5	4·0 ± 0·6	1·1
Intact			
Controls	1·2 ± 0·3	1·5 ± 0·3	1·3
SKF 525-A	2·3 ± 0·4	2·8 ± 0·5	1·2
Hydrocortisone	5·2 ± 0·5	5·9 ± 0·5	1·1

produced only a 20 per cent further increase in measured tryptophan pyrrolase activity when compared with mixtures lacking added hematin. These data indicate that the increases in tryptophan pyrrolase caused by SKF 525-A (and hydrocortisone) are not due to alteration of the "apoenzyme/holoenzyme" ratio but are apparently due to an increase in apoenzyme formation.

Finally, three known inhibitors of tryptophan pyrrolase induction[38, 39] were used in an attempt to disclose an inductive process (i.e. an increase in the rate of apoenzyme formation *de novo*) in the stimulation of tryptophan pyrrolase by SKF 525-A. Graded doses of actinomycin D from 0·1 to 0·5 μmole = 125–625 μg/kg, were co-administered, i.p., with SKF 525-A (100 μmole/kg). The stimulation of tryptophan pyrrolase by SKF 525-A was blocked by actinomycin D at the higher doses (Fig. 3, bottom), suggesting that the stimulation of hepatic tryptophan pyrrolase by SKF 525-A requires synthesis of new mRNA. Similarly, cycloheximide and puromycin abolished the stimulation of tryptophan pyrrolase by SKF 525-A (Fig. 3, top). Cycloheximide was injected once, along with SKF 525-A, at a dose of 2 μmole = 560 μg/kg. Puromycin was given along with SKF 525-A and 2 hr later, at doses of 100 μmole = 54

mg/kg. These inhibitory effects of cycloheximide and puromycin‡ are suggestive of the need for functional translational processes in the stimulating effect of SKF 525-A on hepatic tryptophan pyrrolase.[40]

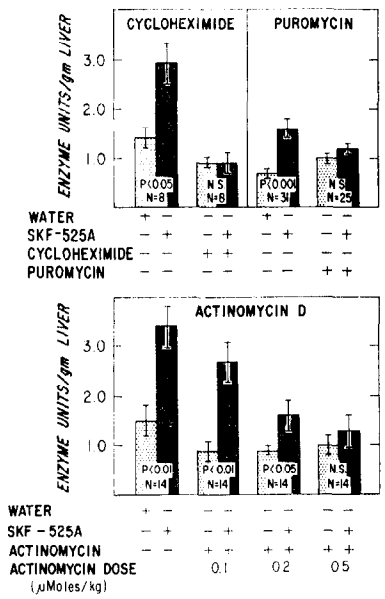

FIG. 3. Effect of actinomycin D, puromycin, and cycloheximide on stimulation of tryptophan pyrrolase in intact rats. The stippled bars represent hepatic tryptophan pyrrolase activity in control animals which received water (10 ml/kg, i.p.) or the antibiotics alone. Hatched bars represent animals which received SKF 525-A either alone or, as indicated, concomitantly with one of the antibiotics. The dose of cycloheximide used was 2 μmole/kg (560 μg/kg) given only at zero time. Puromycin was administered at zero time in a dose of 100 μmole/kg (54 mg/kg) and again at 2 hr. Actinomycin D was given only once in the dosages indicated (0·1–0·5 μmole/kg = 125–625 μg/kg). SKF 525-A was administered in a dose of 100 μmole/kg. All agents were given i.p. Livers were removed 4 hr from zero time and frozen for assay of tryptophan pyrrolase. All animals weighed approx. 200 g except those treated with puromycin; these rats were in the weight range 90–110 g. Statistical significance of the difference between control and SKF 525-A-treated animals, and the total number of animals per group (N) is indicated at the bottom of each double bar. Vertical bars represent ±S.E. Treatment regimen is indicated by plus (+) signs at the bottom of the figures.

Response of rat liver tryptophan pyrrolase to SKF 525-A during maximal induction by hydrocortisone; A study of the mechanism of SKF 525-A induction of tryptophan pyrrolase

As a working hypothesis, we proposed that SKF 525-A might induce tryptophan pyrrolase by the same mechanism as hydrocortisone. Schimke *et al.* reported[42, 43]

‡ Our initial experiments with puromycin were performed in adrenalectomized animals. The dose of SKF 525-A used routinely (100 μmole/kg, i.p.) was lethal to 20 per cent of adrenalectomized animals (five experiments) within 4 hr after receiving SKF 525-A alone. This dose is approximately one quarter of the published LD_{50} dose (i.p.) in intact rats[1] and was not toxic in intact animals receiving SKF 525-A. When both SKF 525-A and puromycin were administered as described above to adrenalectomized rats, nearly 100 per cent lethality resulted within 3 hr. That SKF 525-A has been shown to block *N*-demethylation of puromycin *in vitro*[41] might account for the additive toxicity and explain why puromycin at this comparatively low dose was effective in blocking the increase in tryptophan pyrrolase activity in rats receiving SKF 525-A. Lethal effects of SKF 525-A at a dose of 100 μmole/kg, i.p., were not seen in adrenalectomized animals also receiving hydrocortisone.

that repeated administration of hydrocortisone (every 4 hr) produced a leveling-off of hepatic tryptophan pyrrolase activity in adrenalectomized rats which was evident 8 hr after the first dose of hydrocortisone. Therefore, if SKF 525-A ultimately acted

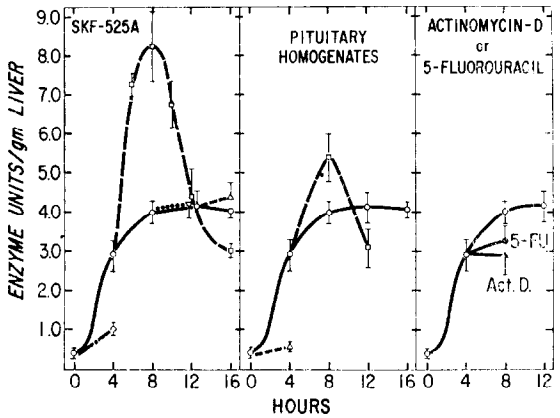

FIG. 4. Effects of administration of SKF 525-A and other agents to adrenalectomized rats undergoing maximal induction of tryptophan pyrrolase after repeated administration of hydrocortisone. Hydrocortisone hemisuccinate (9·6 μmole/rat, subcutaneously, =5 mg) was administered to rats of average weight 155 g (±10 g) at zero time and every 4 hr thereafter. In all panels, data from rats treated only with hydrocortisone are represented by lines connecting the open circles (○——○). In all panels, vertical bars indicate ±S.E., and each point represents an average of nine animals (range: 6–13).

The left panel indicates the changes in tryptophan pyrrolase activity after administration of SKF 525-A (100 μmole/kg, i.p.) to rats treated with hydrocortisone. The hydrocortisone was given zero time and every 4 hr thereafter. The SKF 525-A was administered at zero time or at 4, 8, or 12 hr after the first dose of hydrocortisone. The dashed line going to the diamond-shaped symbol (○—·—◇) represents data from animals injected only with SKF 525-A at zero time. The dashed line going to and connecting the square symbols (□- - -□) represents data from animals receiving hydrocortisone at 0, 4, 8 and 12 hr and also injected with SKF 525-A at 4 hr. The dotted line going to the inverted triangle (· · · · ·▽) represents data from animals treated with hydrocortisone at 0, 4, and 8 hr and also injected with SKF 525-A at 8 hr. The dashed line going to the triangle (– – – –△) represents data from animals treated with hydrocortisone at 0, 4, 8, and 12 hr and also injected with SKF 525-A at 12 hr.

The center panel indicates the changes in tryptophan pyrrolase activity obtained after administration of pituitary homogenates to rats also treated with hydrocortisone. The pituitary homogenates were administered at zero time or at 4 hr after the first dose of hydrocortisone. Each rat injected with pituitary homogenate received the equivalent of two pituitary glands prepared from glands removed from normal rats and frozen on dry ice approximately 30 min before. The homogenates were warmed to room temperature just before injection. The dashed line going to the triangle (– – – –△) represents data from animals injected only with pituitary homogenates. The dashed line going to and connecting the square symbols (– – – –□) represents data from animals receiving hydrocortisone at 0, 4, and 8 hr and also injected with pituitary homogenates at 4 hr.

The right panel indicates the effect of administration of actinomycin D (0·5 μmole/kg, i.p.) or 5-fluorouracil (1 m-mole = 262 mg/kg, i.p.) to animals also treated with hydrocortisone. The actinomycin D and 5-fluorouracil were given 4 hr after the initial dose of hydrocortisone and along with a second dose of hydrocortisone. The 5-fluorouracil was given a second time, 6 hr after the first dose of hydrocortisone, and at this time the dose was also 1 m-mole/kg.

at the same site as hydrocortisone, e.g. on RNA polymerase,† [24, 44–47] then administration of SKF 525-A after the time of maximal stimulation of tryptophan pyrrolase by hydrocortisone should be without effect. This was seen (Fig. 4, left) when SKF 525-A was administered to adrenalectomized animals which had received at least

two doses of hydrocortisone (9·6 μmole = 5 mg hydrocortisone hemisuccinate per rat, subcutaneously, every 4 hr).

However, when SKF 525-A was given at the midpoint of the hydrocortisone–tryptophan pyrrolase-induction phase and along with a second dose of hydrocortisone (i.e. at time = 4 hr), a marked increase of tryptophan pyrrolase activity was obtained. This excessive tryptophan pyrrolase activity rapidly returned to the level of maximally stimulated (by hydrocortisone alone) tryptophan pyrrolase activity at time = 12 hr. This marked potentiation by SKF 525-A of hydrocortisone induction of tryptophan pyrrolase is hereafter referred to as "hydrocortisone de-attenuation" and refers to the ability of SKF 525-A to prevent temporarily the "attenuation" of the enzyme response to hydrocortisone (Fig. 4, left).

Such a de-attenuation by SKF 525-A of hydrocortisone stimulation of tryptophan pyrrolase seems to resemble that reported by Garren *et al.*[48, 49] wherein actinomycin D and canavanine produced marked increases in hepatic tryptophan pyrrolase activity when given after a single dose of hydrocortisone. Garren *et al.* suggested that "attenuation" of the induction phase of tryptophan pyrrolase after hydrocortisone was due to formation of a latent tryptophan pyrrolase "repressor", and that the formation of this repressor after hydrocortisone administration could be inhibited by actinomycin D or canavanine. If this concept were operative in our experiments, then hydrocortisone "deattenuation" caused by SKF 525-A would be due to SKF 525-A acting as a "de-repressor" or affecting the availability of such a mediator.

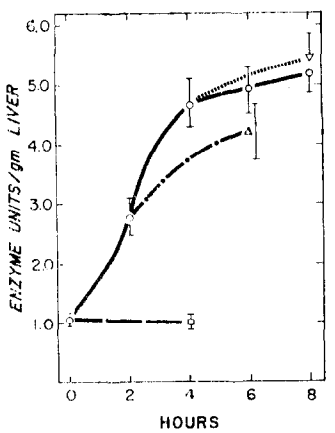

FIG. 5. Effects of administration of SKF 525-A and other agents to hypophysectomized rats undergoing maximal induction of tryptophan pyrrolase after repeated administration of hydrocortisone. This experiment was conducted in a manner analogous to that described under Fig. 4, with the exception that these animals were hypophysectomized. SKF 525-A (100 μmole/kg, i.p.) was administered to rats also treated with hydrocortisone hemisuccinate (9·6 μmole/rat, subcutaneously). The hydrocortisone was given at zero time and every 4 hr thereafter. Data from rats treated only with hydrocortisone are represented by the line connecting the open circles. The dashed line going to the square (– – – –□) represents data from hypophysectomized animals treated only with SKF 525-A at zero time. The dashed line going to the triangle (— – — –△) represents data from hypophysectomized animals treated with hydrocortisone at zero time and 4 hr and also injected with SKF 525-A at 2 hr. The dotted line going to the inverted triangle (· · · ·▽) represents data from hypophysectomized animals treated with hydrocortisone at zero time and at 4 hr and also injected with SKF 525-A at 4 hr. The average weight of the animals was 155 ± 10 g. Vertical lines indicate ±S.E. Each point is representative of data obtained from an average of eight animals (range: 5–9).

However, we were not able to duplicate the results of Garren *et al.* Actinomycin D and 5-fluorouracil administered midway in the hydrocortisone–tryptophan pyrrolase-induction phase seemed to abolish any further increase in activity (Fig. 4, right). Other investigators,[50, 51] using procedures exactly as described by Garren *et al.*, have also failed to confirm the observations of Garren *et al.*

Administration of SKF 525-A to hypophysectomized rats was without effect (under otherwise identical conditions) when such administration occurred during the induction phase (Fig. 5). This seems to indicate that hydrocortisone de-attenuation by SKF 525-A depends on the presence of an intact pituitary gland. It should be recalled that the pituitary is also needed for the stimulatory action of SKF 525-A on hepatic tryptophan pyrrolase (cf. Table 1). Such results again suggested a pituitary–SKF 525-A interaction, and it was hypothesized that injection of pituitary homogenates in the mid-induction phase after hydrocortisone treatment might simulate administration of SKF 525-A at this time. The data in Fig. 4 (center) indicate that this may be so. Saline homogenates of rat pituitary glands injected at zero time usually showed little effect on hepatic tryptophan pyrrolase activity, but injection of pituitary homogenates (equivalent to two pituitary glands per rat) into adrenalectomized rats after an initial dose of hydrocortisone and along with a second dose of the steroid (i.e. at time = 4 hr), produced a short-lived but significant ($P < 0{\cdot}05$, one-tailed), de-attenuation of tryptophan pyrrolase induction. In other words, pituitary homogenates qualitatively mimicked the effects seen after administration of SKF 525-A at this time.

Evidence for a late anti-inductive action of SKF 525-A *on rat liver tryptophan pyrrolase*

Inasmuch as ultrastructural changes and inhibition of drug-metabolizing enzyme activity in the rat liver may persist for at least 24 hr after administration of SKF 525-A,[15] we attempted to ascertain whether SKF 525-A would affect tryptophan pyrrolase induction by hydrocortisone after the initial stimulatory action of SKF 525-A had disappeared. Pretreatment of intact rats with SKF 525-A, given as a single dose (100 μmole/kg, i.p.) 12 hr before, did not affect the initial rate of tryptophan pyrrolase induction by hydrocortisone but did significantly lower the degree to which the enzyme could be stimulated by this dose of hydrocortisone (Fig. 6). Furthermore, readministration of SKF 525-A to SKF 525-A-pretreated animals was without effect on tryptophan pyrrolase activity measured 4 hr after the second dose of the drug (Table 3). That is, SKF 525-A was seen to block its own stimulating effect on tryptophan pyrrolase. The effect of SKF 525-A pretreatment on substrate stimulation of hepatic tryptophan pyrrolase was shown to be minimal however (Table 3).

It is apparent from these experiments that SKF 525-A will diminish or abolish the capacity of the liver to form tryptophan pyrrolase *de novo* from new mRNA. This is an action which qualitatively resembles that of actinomycin, since actinomycin will also block hydrocortisone—but not tryptophan—induction of tryptophan pyrrolase.[38] This inhibitory action of SKF 525-A was seen approximately 12 hr after its administration.

DISCUSSION

Data presented in this paper indicate that SKF 525-A has several, including opposite, effects on hepatic tryptophan pyrrolase activity in rats. These effects include an

early stimulation of tryptophan pyrrolase, a de-attenuation of hydrocortisone induction of the enzyme, and an anti-inductive effect toward hydrocortisone that occurs approximately 12 hr after administration of SKF 525-A.

The early stimulatory action of SKF 525-A on tryptophan pyrrolase in intact rats may, in part, involve a stress reaction. This is suggested by the depletion of adrenal ascorbate that follows the same time course and dose–response relationship as the

TABLE 3. EFFECT OF PRETREATMENT WITH SKF 525-A ON THE STIMULATION OF TRYPTOPHAN PYRROLASE IN INTACT RATS BY L-TRYPTOPHAN OR RE-ADMINISTERED SKF 525-A

Normal (not pretreated, control) animals received water (10 ml/kg), tryptophan (5 m-mole = 1 g/kg), or SKF 525-A (100 μmole/kg), i.p., and their livers were removed 4 hr after treatment. Pretreated animals were given the same regimen 12 hr after pretreatment with SKF 525-A (100 μmole/kg), i.p. Values are mean ± S.E. Numbers in parentheses represent animals per group.

Treatment	Line no.	Tryptophan pyrrolase (units/g liver)		Statistical significance and values compared
		Normal (column 1)	SKF 525-A-pretreated (column 2)	
Controls	1	1·3 ± 0·2 (10)	1·1 ± 0·2 (10)	line 1 vs. line 2; column 1, $P < 0.05$
SKF 525-A	2	2·4 ± 0·3 (10)	0·96 ± 0·1 (10)	line 1 vs. line 2; column 2 $P > 0.05$
L-Tryptophan	3	11 ± 0·7 (7)	7·5 ± 0·6 (7)	line 3; column 1 vs. column 2, $P < 0.05$

increase in enzyme activity. However. this stress reaction appears mediated by the pituitary only, since SKF 525-A can still produce increases in tryptophan pyrrolase activity in the adrenalectomized rat. The nature of the pituitary factor(s) involved is unclear, but would not seem to be identical with ACTH, somatotrophin, or vasopressin. This follows from reports that these pituitary hormones, which are capable of stimulating tryptophan pyrrolase activity in intact rats, are inactive in adrenalectomized animals.[46, 52]

SKF 525-A is also able to de-attenuate hydrocortisone-induced increases in tryptophan pyrrolase. This de-attenuation may be related to the early stimulatory action of SKF 525-A on tryptophan pyrrolase, since both effects require the presence of a pituitary. It was of particular interest to us that the de-attenuative effects of SKF 525-A on hydrocortisone induction of tryptophan pyrrolase could be qualitatively reproduced by injection of crude pituitary homogenates. Again, the nature of the pituitary mediator(s) is unknown but might, in this case, be identical with known hormones like vasopressin. This is true because experiments have not been conducted wherein such possible de-attenuation by known pituitary hormones would be detected.

The stimulatory effects of SKF 525-A on tryptophan pyrrolase are followed, approximately 12 hr later, by an anti-inductive action. At such times, SKF 525-A-pretreated rats are unable to respond maximally to hydrocortisone induction of

tryptophan pyrrolase, and a second injection of SKF 525-A has no stimulatory effect on tryptophan pyrrolase. The carbon tetrachloride-like action of SKF 525-A on the hepatic endoplasmic reticulum[14, 15, 53–55] may be the cause of these late anti-inductive effects of SKF 525-A. SKF 525-A may disrupt ribosome-membrane relationships of the endoplasmic reticulum, decreasing the number of membrane-related ribosomes, thereby reducing the number of sites available for formation of new enzyme from new mRNA. The initial rate of enzyme formation under such circumstances need not be affected; only the total enzyme formed would be reduced. Fig. 6 shows just such a result.

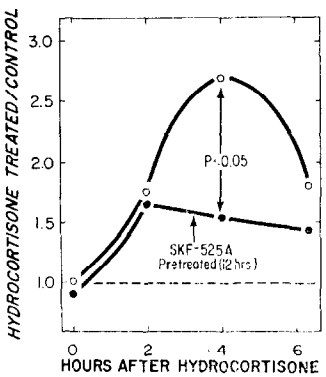

FIG. 6. The effect of 12-hr pretreatment with SKF 525-A on the ability of hydrocortisone to induce hepatic tryptophan pyrrolase. The ordinate represents the ratio of tryptophan pyrrolase activity in animals receiving hydrocortisone to that in control animals. Control animals received water (10 ml/kg, i.p.). The time after hydrocortisone administration is represented on the abscissa. Hydrocortisone (10 μmole hydrocortisone phosphate = 5 mg/kg, i.p.) or water (controls) was administered to intact animals and to those which were pretreated with SKF 525-A. SKF 525-A was given 12 hr previously at a dose of 100 μmole/kg, i.p. Each point represents data from an average of twenty-four rats (range: 16–28). The probability value indicated was obtained from a total of three experiments (5 degrees of freedom, sixteen rats per experiment) wherein the total number of observations (mean ratio of enzyme activity) was 6 at the 4-hr point. Zero-time animals were killed immediately after injection of hydrocortisone (or water). Tryptophan pyrrolase activity at zero time was the same (units/g liver) regardless of treatment or pretreatment.

The late anti-inductive effects of SKF 525-A, as well as its de-attenuative actions on hydrocortisone induction of tryptophan pyrrolase, are at least vaguely reminiscent of actinomycin D effects. Thus, actinomycin D can block hydrocortisone- but not tryptophan-induced increases in tryptophan pyrrolase and, as has been reported by Garren *et al.*,[48, 49] de-attenuate hydrocortisone-induced changes in the enzyme when given during the early phases of induction.

SKF 525-A appears to be similar to hydrocortisone in producing the early stimulation of tryptophan pyrrolase. Both hydrocortisone and SKF 525-A are effective in adrenalectomized animals, and both inductions can be blocked by actinomycin D, cycloheximide, puromycin, and pretreatment (12 hr previously) with SKF 525-A.

SKF 525-A can therefore exhibit certain properties of both hydrocortisone and actinomycin D. Such results should serve as a caution to those who regard SKF 525-A as a specific inhibitor of certain hepatic microsomal mixed-function oxidases—e.g. steroid or drug-hydroxylating enzymes. On these microsomal systems, SKF 525-A

also has apparently opposite effects—an early inhibition and a late inductive effect. The relationships between tryptophan pyrrolase and microsomal enzymes are unclear but might be worth further study.

Acknowledgements—The authors are grateful to the following persons who made drugs available for these studies: Drs. C. A. Stone and G. Boxer of Merck & Co.; Dr. S. S. Stubbs of the Upjohn Co.; Mr. T. Flanagan of Smith Kline & French Inc.; Dr. H. B. Wood of the Cancer Chemotherapy Screening Center. The capable technical assistance of Mr. Douglas Rickert, Mr. Robert Tarvin and Mrs. Roberta Pohl in various aspects of this work is appreciated.

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