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ACTIVATION OF HYDROCORTISONE-INDUCED TRYPTOPHAN PYRROLASE OF RAT LIVER BY SKF 525-A

THE POSSIBLE INVOLVEMENT OF ANTIDIURETIC HORMONE*

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Abstract—The nature and mechanism of the pituitary-dependent potentiation by SKF 525-A of hydrocortisone induction of hepatic tryptophan pyrrolase in the rat was studied. SKF 525-A was found to produce modest increases in plasma corticosterone, a finding which supports previous evidence suggesting that the drug evoked ACTH release. ACTH administration to hydrocortisone-treated rats, however, did not potentiate the action of hydrocortisone on tryptophan pyrrolase. Growth hormone was also inactive in this regard.

Epinephrine, glucagon and both exogenous and endogenous (released by nicotine) antidiuretic hormone were capable of potentiating hydrocortisone induction of tryptophan pyrrolase. Potentiation of hydrocortisone action by SKF 525-A, epinephrine or nicotine was not detectable if enzyme preparations were assayed with excess hematin added *in vitro*, indicating that potentiation occurs via an activation process (as opposed to enzyme induction).

SKF 525-A‡ WAS FOUND to produce in rats a pituitary-dependent enhancement of hepatic tryptophan pyrrolase (EC 1.13.1.12) activity within 4 hr of i.p. administration.[1] The enhanced hepatic tryptophan pyrrolase activity which resulted from SKF 525-A treatment in intact rats was abolished by agents (e.g. actinomycin D, puromycin, cycloheximide) known to prevent glucocorticoid induction of this enzyme.[1]

A peculiar action of SKF 525-A was observed upon administration of the drug to adrenalectomized rats undergoing repeated administration of hydrocortisone. Under these conditions, SKF 525-A markedly enhanced the rate of increase of tryptophan pyrrolase activity over that obtained in rats receiving hydrocortisone alone.[1] However, this "potentiating" action of SKF 525-A was detectable only during the first 8-hr period after hydrocortisone administration and was not obtained when the drug was administered after the inducing action of hydrocortisone was attentuated.[1] Moreover, this hydrocortisone-potentiating action of SKF 525-A was not seen in hypophysectomized animals.[1] This evidence for a pituitary involvement in the action of SKF

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‡ List of abbreviations: SKF 525-A, 2′-diethylaminoethyl-2,2-diphenylpentanoate HCl; ACTH, adrenocorticotropic hormone; STH, somatotropic (growth) hormone; ADH, antidiuretic hormone (vasopressin); cAMP, 3′,5′-cyclic adenosine monophosphate.

525-A on tryptophan pyrrolase was further supported by our finding that injected pituitary homogenates could mimic, at least qualitatively, the hydrocortisone-potentiating action of SKF 525-A.[1]

The experiments described in the present paper were performed in an attempt to identify the nature of and mechanism of action of the pituitary component(s) involved in the potentiating action of SKF 525-A on hydrocortisone induction of hepatic tryptophan pyrrolase

METHODS AND MATERIALS

Animals. All animals used in these experiments were male, Sprague–Dawley rats and were maintained on Purina chow diet and tap water. Rats used to obtain the data in Fig. 1 weighed 280–325 g and came from Simonsen Laboratories (Minneapolis, Minn.). Hypophysectomized rats and their controls used to obtain the data in Figs. 2 and 3 weighed 140–170 g and came from Hormone Assay Laboratories (Chicago, Ill.). Rats used to obtain the data in Figs. 4 and 5 and in Table 1 weighed 140–170 g and were supplied by Simonsen Laboratories. We performed the adrenalectomies under sodium pentobarbital anesthesia (40–50 mg/kg). The diet of adrenalectomized rats was supplemented with 5% glucose and 1% NaCl added to the drinking water. Animals were used 4 days after adrenalectomy. The diet of hypophysectomized rats was supplemented with oranges and the drinking water contained 5% glucose. These animals were shipped 1 day after hypophysectomy and were used 24 hr after their arrival at our laboratory. Total time between surgery and sacrifice was 3–5 days.

In all cases, the control and treated animals in any one experiment were the same strain from the same supplier, the same sex, and approximately the same age and body weight. Both control and treated animals were subjected to the same manipulations (handling and injection either with test drug or solvent) at the same time, and were killed at the same time of day (usually early in the day).

Assay Procedures. Tryptophan pyrrolase was assayed by a modification of the method of Knox[2] as follows: Animals were killed by a blow on the head, and the livers were removed, blotted and quickly frozen on dry ice in prechilled aluminum weighing pans.

Livers for assay were thawed and homogenized in 3 volumes of ice-cold 0·15 M KCl containing 2·5 mM NaOH and 1 mM Na_2EDTA.[3] After 20 min of centrifugation at 9000 *g* in the cold, 0·5 ml or 1 ml (depending on the expected activity of the enzyme) of 9000 *g* supernatant fraction equivalent to 125 or 250 mg liver was incubated in triplicate. Incubation was carried out at 37° with shaking under oxygen for a period of 80 min. The phosphate-buffered incubation medium (0·125 M, pH 7·0) contained ascorbate, 10 mM, and L-tryptophan, 3 mM.[4] After 20 min of incubation, one beaker from each group of three incubated was deproteinized with 15% *meta*phosphoric acid; this beaker served as the incubation blank. The remaining two beakers from each group of three were incubated further for a total of 80 min and then similarly deproteinized. The difference in optical density at 365 mμ of the neutralized, acid-soluble supernatant fractions from the 20-min vs. 80-min incubations (i.e. during the linear portion of the activity vs. time curve) was taken as an index of the enzymic formation of kynurenine (λ_{max}, 365 mμ). Each unit of enzyme catalyzes the formation of 1 μmole kynurenine (via *N*-formylkynurenine) per 60 min under the conditions of incubation. In experiments where total apparent apoenzyme was to be assayed,

hematin (prepared immediately before use[5]) was added in a final optimal concentration of 10^{-5} M.

Corticosterone content of quickly-frozen, heparinized rat plasma (obtained by centrifugation of whole blood drawn by cardiac puncture with a heparinized syringe) and of quickly frozen rat adrenal glands was assayed by the Givner and Rochefort modification[6] of the fluorometric method of Guillemin *et al.*[7]

The following drugs (with their sources in parentheses) were used: hydrocortisone 21-hemisuccinate monosodium (The Upjohn Co.); nicotine tartrate (K & K Laboratories, Inc.); SKF 525-A (Smith, Kline & French, Inc.); aminophylline (G. D. Searle & Co.); ACTH (Corticotropin USP, Parke Davis & Co.); STH (human growth hormone, Raben type, Nutritional Biochemicals); ADH (Pitressin, aqueous, or Pitressin tannate in peanut oil, Parke Davis & Co.); L-epinephrine (Adrenalin Chloride, Parke Davis & Co.); and glucagon HCl (Eli Lilly & Co.).

RESULTS

Effect of SKF 525-A on plasma and adrenal corticosterone levels. We have previously shown that SKF 525-A reduced adrenal ascorbate content by approximately 30 per cent in intact animals.[1] Hypophysectomized animals, however, showed no alteration in adrenal ascorbate content after treatment with SKF 525-A. These findings suggested pituitary activation (ACTH or ADH release) resulting from administration of SKF 525-A. A reliable index of ACTH release by drugs can be obtained by measurement of changes in plasma corticosterone, since such changes are both temporally and quantitatively closely related to the degree of ACTH hypersecretion.[8, 9]

Measurements of plasma and adrenal corticosterone content in animals treated with SKF 525-A at a dose of 39 mg/kg (100 μmole/kg) indicate that a modest increase in plasma and adrenal levels of corticosterone was obtained within 4 hr (Fig. 1).

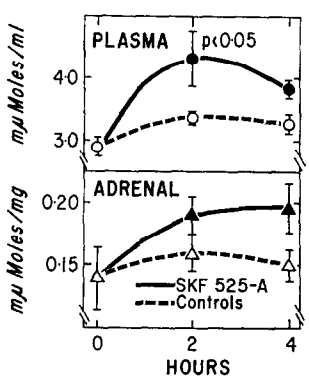

FIG. 1. The effect of SKF 525-A (39 mg/kg, i.p.) on plasma and adrenal corticosterone in adult male rats. Sham-injected control animals were killed and assayed for corticosterone at the same times as those which received SKF 525-A. Data are expressed as mμmoles of corticosterone per ml of plasma or per mg of adrenal tissue from 8 animals per point (mean $\pm$ S.E.).

That only a 25 per cent increase in plasma corticosterone levels was seen indicates that *if* ACTH release was facilitated by SKF 525-A treatment, the amount of ACTH released was relatively minor compared with the ACTH released by agents such as reserpine.[10] While this experiment does not rule out the possibility that SKF 525-A

treatment prolonged the biological half-life of plasma (and adrenal) corticosterone, the results obtained are in agreement with previous evidence suggesting ACTH (or ADH[11–13]) hypersecretion. The observed changes in adrenal ascorbate and corticosterone after SKF 525-A treatment are consistent with pituitary hyperactivity. SKF 525-A does not inhibit ascorbate biosynthesis *in vitro*,[14] and feeding SKF 525-A for protracted periods markedly reduces adrenal corticosterone synthesis.[15] This latter effect may result from inhibition of cholesterol synthesis by SKF 525-A.[16]

Examination of the ability of ACTH, ADH, STH and epinephrine to potentiate hydrocortisone induction of tryptophan pyrrolase. It has been shown that a number of hormones may utilize cAMP as a second "messenger" in their target organs.[17] Tryptophan pyrrolase is activated *in vitro* by a number of chemically unrelated substances. Among these activators is 3′,5′-cyclic adenosine monophosphate (cAMP), as well as other purine (but not pyrimidine) nucleotides.[4, 18–21] Because of this effect of cAMP on tryptophan pyrrolase, a group of pituitary hormones, known either to stimulate tryptophan pyrrolase *in vivo* or known to activate the formation of cAMP in their "target organs" were screened for their ability to mimic the hydrocortisone-potentiating action of SKF 525-A on hepatic tryptophan pyrrolase. Epinephrine, as well, was examined for its ability to potentiate hydrocortisone, since its ability to activate cAMP formation in liver has been well documented.[17, 22]

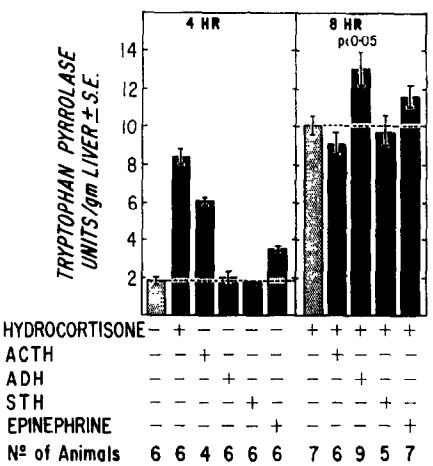

FIG. 2. The effect of selected pituitary hormones or epinephrine on hepatic tryptophan pyrrolase in hypophysectomized vs. hydrocortisone-treated hypophysectomized rats. Hydrocortisone succinate (5 mg/rat) was administered subcutaneously to hypophysectomized rats (weight range, 150–160 g) at zero time or at zero time and again 4 hr later. Tryptophan pyrrolase activity was measured (in the absence of exogenous hematin) in 9000 *g* supernatant fractions from livers of rats killed at the times indicated at the top of each panel. The following hormones were similarly administered, either in the absence of hydrocortisone (left panel) or along with the second injection of hydrocortisone (at 4 hr): ACTH, ADH (aqueous) or STH. Each pituitary hormone was injected s.c. at a dose of 10 units/rat; L-epinephrine was given i.p. in a dosage of 0·5 mg/kg. Four hr after treatment by these hormones, the animals were killed at the time (from zero time) indicated at the top of each panel. A plus (+) sign at the bottom of the figure indicates treatment by the hormones listed. Enzyme activity is expressed as mean enzyme units per gram of tissue ± S.E. Significance values were obtained (Student's *t*-test) from comparison with the mean tryptophan pyrrolase activity in control animals. The dotted lines in each panel indicate the control values which were used to make significance comparisons.

The data in Fig. 2 compare the effectiveness of large doses of ACTH, ADH and STH administered s.c. and epinephrine administered i.p. to mimic the hydrocortisone-potentiating action of SKF 525-A on hepatic tryptophan pyrrolase in hypophysectomized rats. Large, unphysiologic doses of each of these hormones were chosen purposely, primarily because of the rapid degradation of these hormones *in vivo*. Since we were hoping to see effects 4 hr after injection, a choice was made between large single doses and small multiple doses in favor of the former.

Tryptophan pyrrolase activity was increased in hypophysectomized rats by administration of either hydrocortisone or ACTH or by epinephrine (Fig. 2, left panel). The hormones were then administered 4 hr after hydrocortisone treatment and along with a second dose of hydrocortisone, and tryptophan pyrrolase activity was measured 4 hr later (i.e. 8 hr from zero time). Increases in tryptophan pyrrolase activity, i.e. over that in animals receiving 2 doses of hydrocortisone alone, were seen only in those animals which also were given ADH or epinephrine. The potentiating effect of the epinephrine, however, was not statistically significant (Fig. 2, right panel).

Effect of aminophylline on ability of ADH, SKF 525-A and epinephrine to potentiate hydrocortisone. ADH appeared to mimic the potentiating action of SKF 525-A on hydrocortisone induction of hepatic tryptophan pyrrolase. This action of ADH raised the question of the involvement of cAMP in this process. Cyclic-AMP has been implicated in the mediation of ADH control of water movement through otherwise impermeable membranes in nonhepatic tissues.[23] Among the generally accepted criteria for the involvement of cAMP in a physiologic process is that administration of methyl-xanthines (e.g. aminophylline) may potentiate the action of a drug or hormone suspected of acting through cAMP. The underlying requirement for potentiation by a methyl-xanthine is that a first-order relationship prevails regarding cAMP concentration.

Aminophylline pretreatment failed to disclose an effect of ADH on tryptophan pyrrolase in the absence of coadministered hydrocortisone (Fig. 3, left) in hypophysectomized rats. Such pretreatment with aminophylline also failed to potentiate the early stimulating effect of SKF 525-A in adrenalectomized rats (also in the absence of coadministered hydrocortisone; Fig. 4, left panel). Furthermore, pretreatment with aminophylline does not enhance the potentiating action of a reduced dose of ADH in hypophysectomized rats (receiving hydrocortisone as well; Fig. 3, right panel). The hydrocortisone-potentiating action of SKF 525-A in adrenalectomized rats is also unaffected by aminophylline pretreatment (Fig. 4). However, epinephrine appears to produce statistically significant potentiation of hydrocortisone-induced increases in tryptophan pyrrolase in animals which have also received aminophylline (Fig. 2, right panel vs. Fig. 3, right panel). Aminophylline alone has no effect on tryptophan pyrrolase activity.[21]

That neither ADH nor SKF 525-A effects on tryptophan pyrrolase are potentiated by aminophylline does not eliminate the possible involvement of cAMP. Hepatic tryptophan pyrrolase activity, at times when the action of hydrocortisone has been significantly potentiated by ADH, SKF 525-A or by aminophylline with epinephrine, is such that a maximum enzyme activity of the order of 13–15 tryptophan pyrrolase units per gram of liver is obtained (Figs. 2–4). Accordingly, it may be that the process of hydrocortisone potentiation is operating maximally under these conditions. Cyclic AMP involvement, if any, may be such that zero-order (with respect to cAMP

levels) rather than first-order conditions prevail. If this were the case, then aminophylline would not be expected to potentiate a system in which cAMP concentration was already in excess of that necessary to show an effect.

Epinephrine, SKF 525-A, nicotine and glucagon as potentiators of hydrocortisone induction of tryptophan pyrrolase in hematin-activated enzyme preparations. Tryptophan pyrrolase is activated 2-fold or more by the addition *in vitro* of saturating amounts of

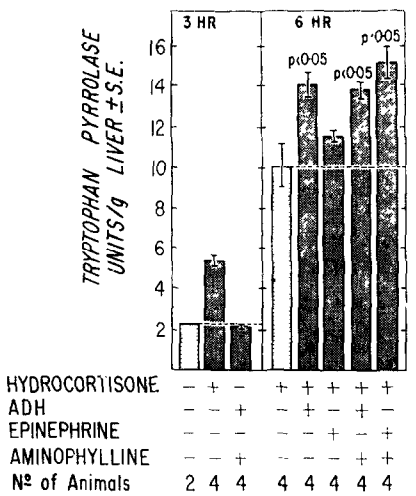

FIG. 3. The effect of aminophylline on ADH or epinephrine potentiation of hydrocortisone induction of hepatic tryptophan pyrrolase in hypophysectomized rats. This experiment is identical to that described under Fig. 2 with the following exceptions: animals were treated with hydrocortisone at 3-hr intervals; the dosage of ADH was reduced to 0·5 unit (subcutaneously, in oil); and aminophylline (45 mg/kg, i.p.) was administered 30 min before zero time (left panel) or 30 min prior to ADH or epinephrine (i.e. at 2·5 hr after the first dose of hydrocortisone) to hydrocortisone-treated rats (right panel).

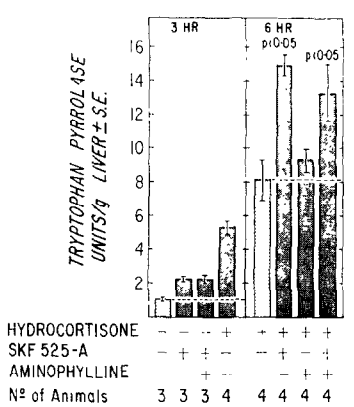

FIG. 4. The effect of aminophylline on SKF 525-A potentiation of hepatic tryptophan pyrrolase induction by hydrocortisone in adrenalectomized rats. This experiment is identical to the one described under Fig. 3, except that in this case the rats were only adrenalectomized and SKF 525-A was administered (39 mg/kg, i.p.) in place of ADH or epinephrine. Aminophylline was injected at a dose of 45 mg/kg as described in the legend to Fig. 3.

hematin or methemoglobin.[4] Hematin added *in vitro* to tryptophan pyrrolase assay flasks has been used by several investigators for the purpose of measuring the degree of conjugation of apotryptophan pyrrolase with its prosthetic group.[24] It was therefore presumed that if the mechanism of potentiation (e.g. by SKF 525-A) of hydrocortisone action involved an induction process, the addition of hematin *in vitro* should disclose the presence of unconjugated apoenzyme.

Adrenalectomized rats were treated like the animals used in previous experiments (Figs. 2–4) except that nicotine was administered in place of ADH. The ADH-releasing property of nicotine[25] makes this latter drug a useful tool in circumventing the problem of using unphysiological doses of ADH (Figs. 2 and 3). Enzyme assays were performed with or without the addition of hematin at a concentration of 10^{-5} M.

It will be seen in Fig. 5 (left panel) that nicotine administered alone produces a slight *activation* of tryptophan pyrrolase ($P < 0{\cdot}05$), i.e. an increase in the enzyme activity

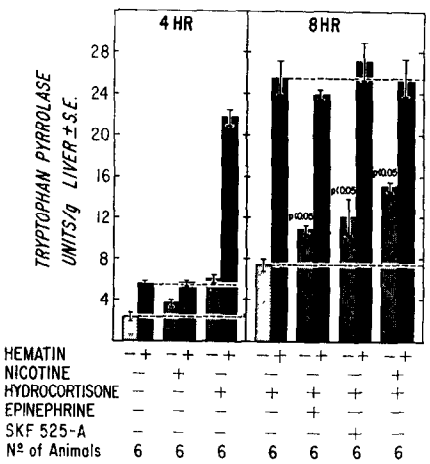

FIG. 5. The effect of hematin addition *in vitro* on the potentiation of hydrocortisone induction of hepatic tryptophan pyrrolase in adrenalectomized rats by nicotine, epinephrine or SKF 525-A. Drugs were injected as described in preceding experiments (Fig. 2). The hematin concentration was 10^{-5} M. The dosage of nicotine tartrate was 2 mg/kg (s.c.) given every 2 hr for a total of 2 doses. The epinephrine dosage was 0·6 mg/kg in oil, s.c.

(holoenzyme) which is detectable only in the absence of added hematin. Hydrocortisone, however, increases enzyme activity in both assay conditions (with or without hematin), as would be expected from earlier reports.[24] It will also be seen that epinephrine (at a dose of 0·6 mg/kg in oil, s.c.), SKF 525-A or nicotine significantly potentiated the action of hydrocortisone on tryptophan pyrrolase. However, this increase in tryptophan pyrrolase activity was not detectable if the enzyme was already maximally activated *in vitro* by hematin.

A similar experiment was performed in intact rats receiving repeated doses of hydrocortisone. In this experiment, however, glucagon[22] was administered 4 hr after the first dose of hydrocortisone and together with a second dose of hydrocortisone. It can be seen (Table 1) that increased enzyme activity assayed 8 hr after the first dose of hydrocortisone (i.e. 4 hr after glucagon) can be detected in the glucagon-treated group, but only in preparations assayed in the absence of exogenous hematin

(holoenzyme). While glucagon has recently been shown to induce hepatic tyrosine transaminase in the rat, it appears to have no effects *in vivo* on hepatic tryptophan pyrrolase, as studied *in vitro* in hematin-activated enzyme assays of liver preparations from control or hydrocortisone-treated animals.[26, 27]

TABLE 1. DIFFERENTIAL ACTION OF GLUCAGON ON HYDROCORTISONE-INDUCED HOLOTRYPTOPHAN PYRROLASE VS. APOENZYME IN INTACT RATS*

Treatments	Time after first injection (hr)	Tryptophan pyrrolase activity (units/g liver ± S.E.)	
		Holoenzyme (— hematin)	Apoenzyme (+ hematin)
No treatment		3·0 ± 0·1	6·0 ± 0·1
Hydrocortisone	4	6·0 ± 0·5	16 ± 1
Hydrocortisone	8	6·3 ± 0·9†	20 ± 2
Hydrocortisone + glucagon	8	8·5 ± 0·3†	21 ± 2

* This experiment was identical to that performed as described previously (Figs. 2 and 5) except that intact rats were used. The dose of glucagon was 1 mg per rat (average weight 140–160 g) i.p., administered to hydrocortisone-treated rats 4 hr after the first dose of hydrocortisone. Each mean represents data from 5 animals. Student's *t*-test was based on a one-tailed distributon.

† Significantly different from each other ($P < 0·05$).

These experiments (Fig. 5, Table 1) suggested that potentiation of hydrocortisone action (by SKF 525-A, etc.) occurred via an activation of the apoenzyme form of tryptophan pyrrolase and was not due to enhanced synthesis *de novo* of this enzyme. The potentiating effect of nicotine on hydrocortisone induction of tryptophan pyrrolase suggests that endogenous ADH may also activate tryptophan pyrrolase. Nicotine may also have acted via nonadrenal catecholamine stores.

DISCUSSION

The present experiments were intended to provide information on the possible nature and mechanism of action of the pituitary component which mediates SKF 525-A potentiation of hepatic tryptophan pyrrolase induction by hydrocortisone. What has not been determined as yet is why potentiation of hydrocortisone action by SKF 525-A occurs only when the latter drug is administered during the initial induction phase *following* hydrocortisone administration.[1] SKF 525-A does not exhibit this potentiating action if administered during the attenuated phase (i.e. steady state phase) of hydrocortisone action.[1] It would be convenient to describe this hydrocortisone potentiation phenomenon in terms of SKF 525-A acting as an antagonist of tryptophan pyrrolase "repressor" formation.[28] However, several laboratories have been unable to confirm the presence of a tryptophan pyrrolase repressor mechanism in attenuation of hydrocortisone action.[27, 29, 30] Such a de-repressor mechanism of potentiation necessarily invokes the involvement of further synthesis *de novo* of apotryptophan pyrrolase above that produced by hydrocortisone alone, i.e. a "superinduction". Such a superinduction has been described for tyrosine transaminase in tissue culture.[31, 32] Since by the addition of hematin *in vitro* we were unable to show the presence of a superinduction, it is concluded that the process of potentiation occurs by activation of pre-existing (hydrocortisone-induced) apoenzyme.

The mechanism of this activation process remains to be elucidated. That SKF 525-A, epinephrine, glucagon and both endogenous and exogenous ADH share this activating property supports the possible involvement of cAMP in the potentiation of tryptophan pyrrolase. However, it remains to be shown: (1) that SKF 525-A and ADH stimulate adenyl cyclase activity in liver; ADH has been shown to stimulate adenyl cyclase in kidney.[33, 34] (2) that SKF 525-A facilitates release of ADH *in vivo*. While SKF 525-A is diuretic when injected into the renal artery,[35, 36] it is markedly antidiuretic when administered i.p. to hydrated rats;[37] while such a latter result could be due to several factors, it could also arise from stimulation of ADH release *in vivo* by the drug. (3) that if adenyl cyclase of liver *is* stimulated by ADH and SKF 525-A, the amount of cAMP that would be present *in vitro* under conditions of assay of tryptophan pyrrolase would account for the marked change in enzyme activity seen.

There is at present only a suggestion, at best, of the involvement of ADH and cAMP in the potentiation by SKF 525-A of hydrocortisone induction of tryptophan pyrrolase. The available data are consistent with such a mechanism. Cyclic AMP and hematin are additive activators of tryptophan pyrrolase *in vitro* when hematin is present in a concentration of 10^{-6} M.[23] In the present studies, the addition of hematin (10^{-5} M) to tryptophan pyrrolase assay flasks *in vitro* failed to disclose the presence of an *additive* activator. From this same experiment, where hematin addition to assay flasks was studied, it would appear that hydrocortisone potentiation by SKF 525-A, ADH, glucagon and epinephrine occurred by a similar mechanism, i.e. activation of apoenzyme. Whether or not cAMP is involved in this process must await further experimentation.

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