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MEASUREMENTS OF TRYPTOPHAN PYRROLASE *IN VIVO*: INDUCTION AND FEEDBACK INHIBITION

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SUMMARY

The level of tryptophan pyrrolase (L-tryptophan:O_2 oxidoreductase, EC 1.13.1.12) is regulated by hormones as well as by its substrate, tryptophan. Recently it has been proposed that this enzyme may also be regulated by feedback inhibition by NAD (ref. 7). We have investigated the validity of this hypothesis for tryptophan pyrrolase operating *in vivo*. The activity of tryptophan pyrrolase *in vivo* was measured by determining the rate of conversion of injected [*ring*-2-^{14}C]tryptophan to $^{14}Co_2$in the rat. The level of hepatic NAD was elevated by injection of nicotinamide. In normal animals injection of nicotinamide evoked a sharp rise in tryptophan pyrrolase activity which was apparently hormone mediated since hypophysectomized animals did not show this increase. In hypophysectomized rats the rate of $^{14}CO_2$ release from tryptophan varied inversely with the hepatic NAD concentration. This finding strongly suggests that NAD may be functioning as a feedback inhibitor of tryptophan pyrrolase *in vivo*.

INTRODUCTION

The enzyme tryptophan pyrrolase (L-tryptophan:O_2 oxidoreductase, EC 1.13.1.12)[1] catalyzes the first step in the series of reactions which leads ultimately to both the conversion of the benzene ring of tryptophan to CO_2 *via* the "glutarate" pathway[2–4] and to its conversion to the nicotonyl moiety of the pyridine nucleotides *via* the NAD pathway[5,6].

Recently, WAGNER[7] suggested that the level of hepatic NAD may regulate the conversion of tryptophan to NAD through feedback inhibition of tryptophan pyrrolase activity (Reaction a). This hypothesis seemed open to question on the following grounds: (a) the level of NAD required to show inhibition of tryptophan pyrrolase *in vitro* was higher ($5 \cdot 10^{-3}$ M) than reported hepatic NAD concentrations (*e.g.* refs. 8, 9); (b) inhibition of tryptophan pyrrolase would depress conversion of

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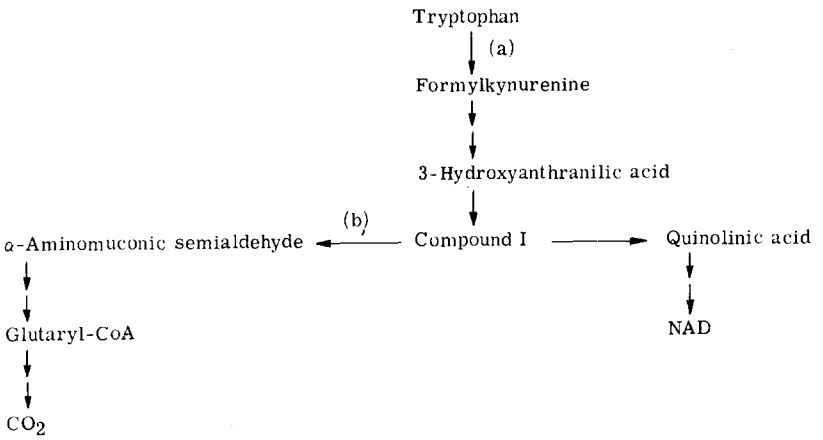

tryptophan by both the NAD and glutarate pathways. *A priori* one would expect control of NAD synthesis to operate at or beyond the branch point of these two pathways; (c) considerable evidence has been educed to show that the conversion of tryptophan to NAD is regulated by the level of picolinic carboxylase (Reaction b)[10–12], an enzyme located at the branch point of the glutarate and NAD pathways and (d) tryptophan pyrrolase is known to be very sensitive to regulation by substrate and hormones (*e.g.* ref. 13).

METHODS

DL-[*ring*-2-^{14}C]tryptophan was purchased from Tracerlab Inc., L-tryptophan from Sigma Chemical Company, hydrocortisone acetate from Mann Research Laboratories and hematin from Calbiochem, Inc. Normal male rats were purchased from Holtzman and adrenalectomized and hypophysectomized rats from Hormone Assay Laboratories, Inc., Chicago, Ill. Rats were not used until at least 5 days after operation and were maintained on laboratory chow with drinking water replaced by 0.85% NaCl for the adrenalectomized and 5% glucose solution for the hypophysectomized animals. DL-[*ring*-2-^{14}C]tryptophan, 1.0 μg (0.65 μC) *plus* varying amounts of L-tryptophan were dissolved in 1 M NaOH and injected intraperitoneally. $^{14}CO_2$ was collected and the radioactivity determined as previously described[14].

Tryptophan pyrrolase was assayed by the method of KNOX AND MEHLER[1] as modified by FEIGELSON AND GREENGARD by the addition of hematin[15]. For the determination of hepatic NAD concentration rats were killed by cervical dislocation and the livers immediately perfused with ice-cold 0.85% NaCl. Subsequent steps in the determination of NAD were carried out as described by GREENGARD, GUINN AND REID[16].

RESULTS

Measurement of tryptophan pyrrolase activity in vivo

The rate of oxidation of a dose of α-[^{14}C]tryptophan or β-[^{14}C]tryptophan has been previously used as an index of the activity of tryptophan pyrrolase *in vivo*[17].

Conversion of these compounds to $^{14}CO_2$ requires the participation of several other enzymes of tryptophan catabolism[18] as well as the enzymes of the tricarboxylic acid cycle[19]. Therefore, this assay would only be valid if tryptophan pyrrolase is the rate-limiting step in an extensive metabolic sequence, and it might be difficult to establish whether a particular treatment was affecting tryptophan pyrrolase activity or a later step in this sequence. The conversion of [*ring*-2-^{14}C]tryptophan to $^{14}CO_2$ involves fewer reactions than does the oxidation of side chain labeled tryptophan. Tryptophan pyrrolase should be the rate-limiting enzyme in the conversion of [*ring*-2-^{14}C]tryptophan to $^{14}CO_2$ since formyl kynurenine formylase is known to be present in very great excess over tryptophan pyrrolase in rat liver[1,13] and since injected [^{14}C]formate is converted to $^{14}CO_2$ much more rapidly than is [*ring*-2-^{14}C]-tryptophan (40–60% in 1 h). Therefore, [*ring*-2-^{14}C]tryptophan was used for assay of tryptophan pyrrolase activity *in vivo*.

The well-known principle that quantitative assays for enzyme activity should be carried out if possible at saturating levels of substrate should also apply to assays *in vivo*. Therefore, various levels of L-tryptophan were injected with constant levels of DL-[*ring*-2-^{14}C]tryptophan (0.65 μC) and the rate of $^{14}CO_2$ excretion was determined. The data in Fig. 1 demonstrate that the per cent of a given dose of tryptophan which is oxidized to CO_2 depends on the dose level and suggest that at the higher levels used the system is approaching saturation with substrate. Increasing the dose from 0.51 to 0.75 mg/g resulted in very little change in the absolute rate of $^{14}CO_2$ formation in the first 5 h following injection while increasing the dose to 1.5 mg/g resulted in a decrease in CO_2 formation during this time. These measurements are complicated by the fact that tryptophan itself causes an increase in total tryptophan pyrrolase[20]. However, 4 h are required for the approximate doubling of tryptophan

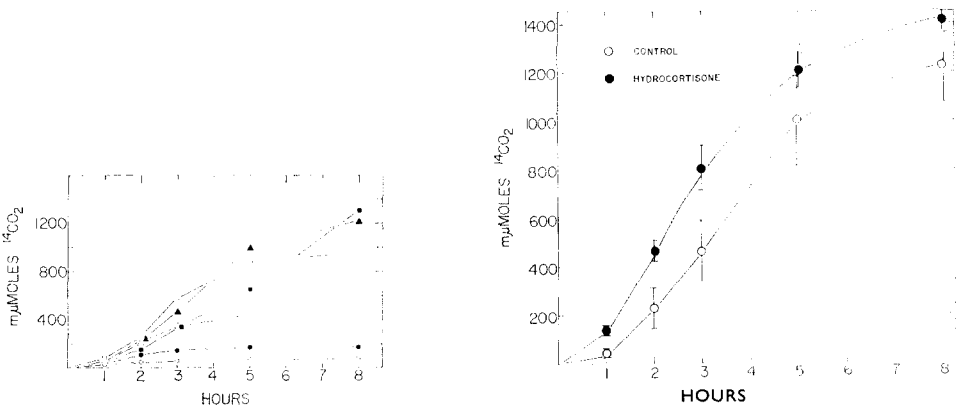

Fig. 1. Time course of $^{14}CO_2$ excretion following injection of [*ring*-2-^{14}C]tryptophan as a function of dose level. Each curve represents an average of two or more animals. Experimental conditions are described in the text. The levels of L-tryptophan injected in mg per g body weight are as follows: 0.075, ○---○; 0.15, ●—●; 0.30, △- -△; 0.51, □ -□; 0.75, ▲—▲; 1.5, ■—■.

Fig. 2. Rate of conversion of [*ring*-2-^{14}C]tryptophan to $^{14}CO_2$ in control and hydrocortisone-treated rats. Control rats, ○---○. Rats injected intraperitoneally with 2.5 mg/100 g hydrocortisone acetate 3 h before tryptophan injection, ●—●. All animals were injected intraperitoneally with 0.75 mg/g [*ring*-2-^{14}C]tryptophan at zero time. Each point on the curve for control rats represents the average of data from four animals. Each point on the curve for hydrocortisone-treated rats represents the average data from three animals. The bars represent the S.E.

pyrrolase activity in response to injection of 1.0 mg/g L-tryptophan[13]. Therefore, the rate of $^{14}CO_2$ release in the early portion of the curve should be due largely to tryptophan pyrrolase activity already present at the time of tryptophan injection. On the basis of the results shown in Fig. 1, a dose level of 0.75 mg tryptophan per g body weight was chosen for further experiments. At this dose level approx. 35% of injected tryptophan was excreted as CO_2 in 8 h.

Injection of adrenocorticoid hormones increases the level of rat-liver tryptophan pyrrolase by 4–10-fold in 4–5 h[21]. If the assay for tryptophan pyrrolase *in vivo* has any validity, pre-treatment of rats with hydrocortisone should cause an increase in the rate of conversion of [*ring*-2-^{14}C]tryptophan to $^{14}CO_2$. The data presented in Fig. 2 show that hydrocortisone administration increased $^{14}CO_2$ formation. Control experiments demonstrated that this effect cannot be due to any influence by hydrocortisone on the rate of formate oxidation. The increased rate of $^{14}CO_2$ excretion therefore may be interpreted as an actual measurement, *in vivo*, of increased tryptophan pyrrolase activity.

Injection of α-methyltryptophan is also known to increase the level of rat-liver tryptophan pyrrolase[17]. When 0.755 mg/g α-methyltryptophan was injected into rats 4 h prior to [*ring*-2-^{14}C]tryptophan (0.075 mg/g) administration, the amount of $^{14}CO_2$ excreted after 1 h was 60% greater than in the control animals receiving to α-methyltryptophan. This finding further supports the validity of the tryptophan pyrrolase assay *in vivo*.

Effect of hepatic NAD concentration on tryptophan pyrrolase

The results presented above suggest that it is possible to measure changes in tryptophan pyrrolase activity *in vivo* and thus to determine the effects, if any, of changes in hepatic NAD levels on tryptophan pyrrolase activity. Several groups of workers[16,22,23] demonstrated that injection of nicotinamide into mice and rats causes a several fold increase in the concentration of hepatic NAD. The influence of elevated hepatic NAD levels on the oxidation of [*ring*-2-^{14}C]tryptophan was tested by injecting 50 mg nicotinamide per 100 g body weight intraperitoneally into rats and 4 h later, when hepatic NAD concentration should be at a maximum[16,23], injecting the test dose of [*ring*-2-^{14}C]tryptophan. The results of these experiments are shown in Fig. 3. In the animals pretreated with nicotinamide the initial rate of $^{14}CO_2$ formation was greater than in the controls but decreased to less than control values after 3–4 h. These results were unexpected and suggest an initial stimulation and subsequent inhibition of tryptophan pyrrolase activity caused by nicotinamide administration. This phenomenon was investigated more closely by determining the time course of tryptophan pyrrolase activity as measured *in vitro* following administration of nicotinamide. The data presented in Fig. 4 show that nicotinamide administration causes a sharp increase in tryptophan pyrrolase activity. This activity decays faster than the elevated NAD level also elicited by nicotinamide administration. The results shown in Fig. 3 may be explained on the basis of the changing levels of tryptophan pyrrolase and NAD presented in Fig. 4. [*ring*-2-^{14}C]Tryptophan was administered 4 h after nicotinamide at a time when tryptophan pyrrolase activity has reached a maximum. This could explain the more rapid initial evolution of $^{14}CO_2$ in the nicotinamide-treated animals. The level of tryptophan pyrrolase decreased rapidly while the level of hepatic NAD remained elevated. Therefore, the decrease

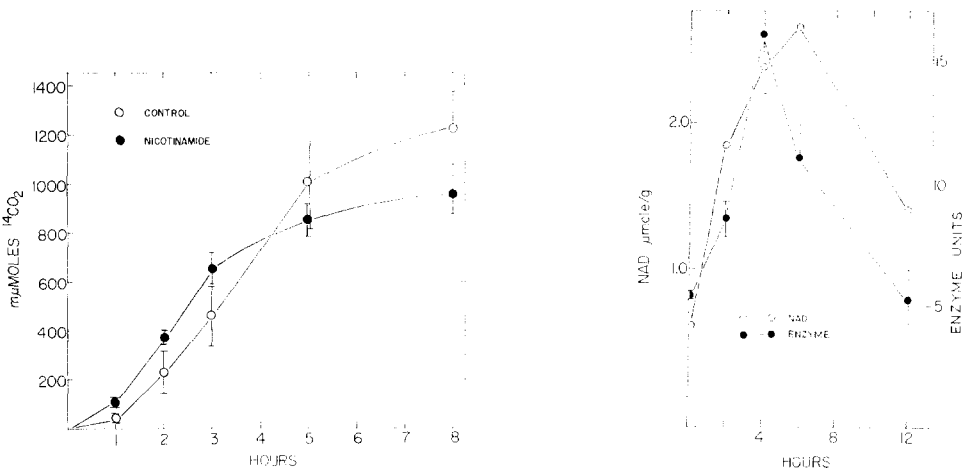

Fig. 3. Effect of pre-injection with nicotinamide on the rate of information of $^{14}CO_2$ from [*ring*-2-^{14}C]tryptophan in normal rats. Curves represent average values for four animals. Controls, ○—○. Rats injected intraperitoneally with nicotinamide (5 mg per 100 g body weight) 4 h prior to tryptophan administration, ●—●. All animals were injected intraperitoneally with 0.75 mg/g [*ring*-2-^{14}C]tryptophan at zero time. The bars represent the S.E.

Fig. 4. Response of hepatic NAD concentration and tryptophan pyrrolase activity following intraperitoneal injection of nicotinamide. At zero time 24 rats were injected intraperitoneally with 50 mg per 100 g body weight nicotinamide. At zero, 2, and 4 h after injection six rats were sacrificed and hepatic NAD concentration (○—○) and tryptophan pyrrolase activity determined (enzyme, ●—●) as described in the text. At the 6- and 12-h intervals three animals were used for these determinations. The bars represent the S.E.

in the rate of evolution of $^{14}CO_2$ in the nicotinamide-treated animals, which occurred 3 h after tryptophan administration, could be explained if NAD is inhibiting tryptophan pyrrolase.

While these studies were in progress, it was brought to our attention that PALLINI, VASCONETTO AND PICSI[24] had reported that intraperitoneal injection of

TABLE I

TRYPTOPHAN PYRROLASE ACTIVITY FOLLOWING NICOTINAMIDE INJECTION OF NORMAL, ADRENALECTOMIZED AND HYPOPHYSECTOMIZED RATS

Tryptophan pyrrolase activity as measured *in vitro* at various time intervals following intraperitoneal injection of 500 mg/kg nicotinamide. Results are expressed as mμmoles kynurenine/h per mg protein. Values are averages ±S.E. Numbers in parentheses refer to number of animals assayed in each group. Control rats injected with 3 ml of saline showed no significant response in tryptophan pyrrolase activity.

Time after nicotinamide injection (h)	*Normal (6)*	*Adrenalectomized (3)*	*Hypophysectomized (3)*
0	5.4 ± 1.2	7.1 ± 1.4	11.2 ± 1.2
2	8.5 ± 1.1	7.7 ± 1.4	9.0 ± 0.7
4	16.0 ± 2.4	5.6 ± 0.3	9.6 ± 2.0
6	11.0 ± 3.2	6.1 ± 1.8	8.2 ± 0.3
12	5.2 ± 1.1	5.7 ± 0.4	7.9 ± 1.0

nicotinamide "induces" tryptophan pyrrolase in the livers of normal but not adrenalectomized rats. This action of nicotinamide in elevating the level of tryptophan pyrrolase is reminiscent of the non-specific stimulation of tyrosine-α-ketoglutarate transaminase by the injection of a variety of substances[25,26], a phenomenon known to be mediated through adrenal hormones. Therefore, the effect of nicotinamide administration on tryptophan pyrrolase was tested in adrenalectomized and hypophysectomized rats with the results shown in Table I. In these animals there was no increase in the activity of tryptophan pyrrolase as measured *in vitro* following intraperitoneal injection of 500 mg/kg nicotinamide. These findings suggest that the intact pituitary as well as adrenals are necessary for the nicotinamide evoked increase in tryptophan activity observed in normal animals.

Nicotinic acid is an even more potent inducer of tryptophan pyrrolase than is nicotinamide as shown by the data in Table II. An intraperitoneal injection of

TABLE II

TIME RESPONSE OF TRYPTOPHAN PYRROLASE ACTIVITY TO INJECTION OF 500 MG/KG NICOTINIC ACID OR NICOTINAMIDE

Values for nicotinamide injection are averages of 5 animals and for nicotinic acid of 2–4 animals. Experimental details in the text.

Time after nicotinamide injection (h)	*Tryptophan pyrrolase activity (mμmoles kynurenine/h per mg protein)*	
	Nicotinic acid	*Nicotinamide*
0	5.4	5.4
2	12.3	8.5
4	28.0	16.0
6	36.8	11.0
8	32.0	5.2
12	20.6	—
24	19.0	—

500 mg/kg of nicotinic acid produced a higher and more prolonged rise in tryptophan pyrrolase activity than did an equal amount of nicotinamide. Nicotinic acid is also more effective than nicotinamide at lower dose levels as shown by the dose response data in Table III. As in the case of nicotinamide, induction of tryptophan pyrrolase by nicotinic acid did not occur in hypophysectomized animals.

Adrenalectomized rats exhibit a more prolonged rise in hepatic NAD levels following nicotinamide administration than do normal animals but the maximum NAD levels reached are approximately the same as in normal rats[27]. However, the NAD levels attained in the livers of hypophysectomized rats in response to nicotinamide are about twice those of the normal animal[16]. Therefore, if hepatic NAD inhibits tryptophan pyrrolase *in vivo*, this effect should be particularly pronounced in hypophysectomized rats injected with nicotinamide. The results presented in Fig. 5 show that conversion of [*ring*-2-^{14}C]tryptophan to $^{14}CO_2$ in hypophysectomized rats is greatly inhibited by prior injection of nicotinamide. There was no indication

TABLE III

TRYPTOPHAN PYRROLASE ACTIVITY 4 H AFTER ADMINISTRATION OF DIFFERENT LEVELS OF NICOTINAMIDE AND NICOTINIC ACID

Values for nicotinamide injection are averages of 5 animals and for nicotinic acid injection of 2 animals. Experimental details in the text.

Compound administered	*Dosage (mg/kg)*					
	0	*100*	*200*	*500*	*750*	*1000*
	mμmoles kynurenine/h per mg protein					
Nicotinamide	5.4	—	11.6	21.5	31.2	28.8
Nicotinic acid	—	11.1	27.2	27.0	29.0	—

of an increase in tryptophan pyrrolase activity as seen in normal animals (Fig. 3). Control experiments demonstrated that nicotinamide injection had only a slight inhibitory effect on the oxidation of sodium formate to CO_2 in hypophysectomized rats.

The results presented in Fig. 5 strongly suggest that prior administration of nicotinamide exerts an inhibitory effect upon the activity of tryptophan pyrrolase *in vivo*. However, they do not establish whether this inhibition is due to increased concentrations of NAD or to some other effect of nicotinamide administration. If NAD is acting to regulate tryptophan pyrrolase activity *in vivo* then one might

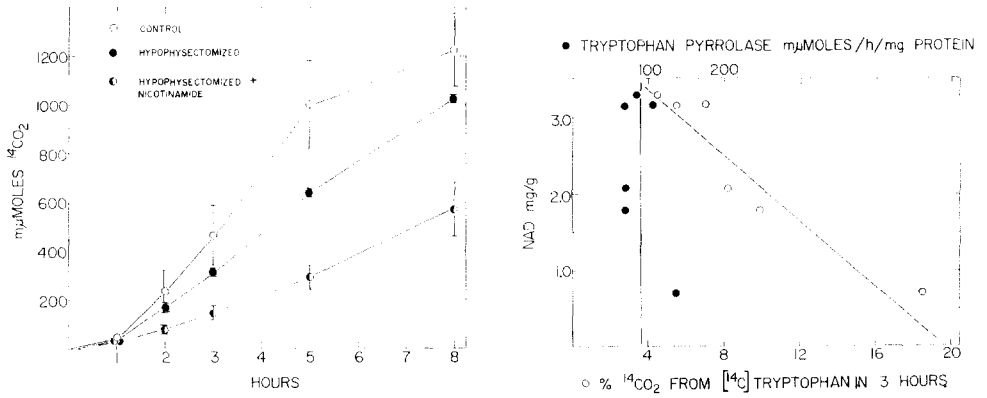

Fig. 5. Effect of pre-injection with nicotinamide on the rate of formation of $^{14}CO_2$ from [*ring*-2-^{14}C]tryptophan in hypophysectomized rats. Curves represent average values for four normal and hypophysectomized animals. Control rats, ○—○. Hypophysectomized rats, ●—●. Hypophysectomized rats injected intraperitoneally with nicotinamide (50 mg per 100 g body weight) 4 h prior to tryptophan administration, ◐—◐. All animals were injected intraperitoneally with 0.75 mg/g [*ring*-2-^{14}C]tryptophan at zero time. The bars represent the S.E.

Fig. 6. Correlation of hepatic NAD concentration and tryptophan pyrrolase activity *in vivo* in hypophysectomized rats. Hypophysectomized rats were injected with 50 mg per g body weight of nicotinamide and at zero time, 30 min, 6 h, 12 h and 24 h, three animals were injected intraperitoneally with 0.75 mg/g [*ring*-2-^{14}C]tryptophan and expired $^{14}CO_2$ collected for 3 h. The animals were then sacrificed and hepatic NAD concentrations determined *inv itro* for 1 or 2 animals in each group. The values for each group were averaged. % of $^{14}CO_2$ released from [*ring*-2-^{14}C]tryptophan in 3 h, ○—○. Tryptophan pyrrolase activity of the same group of animals as measured *in vitro*, ●—●.

expect an inverse relationship between the hepatic level of NAD and the rate of $^{14}CO_2$ production from [*ring*-2-^{14}C]tryptophan. Since the concentration of hepatic NAD in hypophysectomized rats injected with nicotinamide changes slowly over a wide range of values[16] it is possible to obtain animals with relatively constant hepatic NAD concentrations which differ from each other by as much as 300%. When the amount of $^{14}CO_2$ excreted in 3 h after injection of [*ring*-2-$^{14}CO_2$]tryptophan was plotted against the hepatic NAD concentrations of hypophysectomized rats at the end of the same 3-h period, the results shown in Fig. 6 were obtained.

When the concentration of hepatic NAD was approx. 3 mg/g 5–7% of the injected [*ring*-2-^{14}C]tryptophan was excreted as $^{14}CO_2$ in 3 h, when the level of NAD was about 2 mg/g 8–10% of the ^{14}C was excreted as $^{14}CO_2$ in 3 h and when the concentration of NAD was below 1 mg/g about 18% of the injected tryptophan was converted to $^{14}CO_2$ in the 3-h period. Prior injection of nicotinic acid into hypophysectomized rats also caused a marked inhibition of the conversion of [*ring*-2-^{14}C]-tryptophan to $^{14}CO_2$ with a concomittant increase in hepatic NAD concentration. In contrast to the assay *in vivo* the hepatic tryptophan pyrrolase activity as determined *in vitro* on the same liver samples (solid circles in Fig. 6) showed no appreciable changes with NAD concentration. These results suggest that at the levels of NAD concentration investigated tryptophan pyrrolase activity *in vivo* bears an inverse relationship to hepatic NAD concentration. It would therefore appear that NAD is acting as a feedback inhibitor of tryptophan pyrrolase *in vivo*.

DISCUSSION

The results presented in this paper strongly support the proposal of WAGNER[7] that NAD may be a feedback inhibitor of tryptophan pyrrolase. Tryptophan pyrrolase initiates the sequence of reactions leading to the complete oxidation of the benzene ring of tryptophan, as well as the pathway leading to NAD (ref. 18). However, under conditions of high NAD levels it may be necessary for the animal to reduce the flow of tryptophan through both pathways in order to decrease NAD formation. It would be more logically satisfying to regulate the formation of NAD by feedback inhibition, at, or past, the branch point; however, the rat does not appear to have developed regulatory enzymes at these steps.

The previous suggestion that NAD may be a feedback inhibitor of the enzyme which converts quinolinic acid to nicotinic acid mononucleotide[6] has been found to be erroneous. The inhibition of this enzyme by NAD observed in crude preparations is due to competition for 5-phosphoribosyl 1-pyrophosphate by degradation products of NAD*.

Induction of tryptophan pyrrolase by tryptophan[13] would operate in an inverse sense to the inhibition exerted by NAD. The combined operation of these opposing mechanisms may insure a regulated flow of tryptophan to NAD. Regulation of conversion of tryptophan to NAD by control of the level of picolinic carboxylase appears to be a long-term hormonally mediated effect[10–12] in contrast to the rapid control provided by feedback inhibition. Therefore, both mechanisms may be essential in controlling the rate of NAD synthesis.

* M. SHIMOYAMA AND R. K. GHOLSON, unpublished observations.

The data presented above are also consistent with the hypothesis that nicotinamide and nicotinic acid "induce" tryptophan pyrrolase by causing increased secretion of adrenal cortical hormones. Since hypophysectomized animals do not show this "induction", this response is probably mediated through the pituitary. This effect is probably due to free nicotinamide or nicotinic acid rather than to an indirect effect on NAD biosynthesis because, at high dose levels, injected nicotinamide is much more potent than nicotinic acid in causing increased NAD biosynthesis[23,28,29] but nicotinic acid is more active than nicotinamide in inducing tryptophan pyrrolase activity. In fact, the tryptophan pyrrolase inducing activity of nicotinamide may be due to its conversion *in vivo* to nicotinic acid by the enzyme nicotinamide deamidase[30].

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