The Effects of Acute and Chronic Nicotine Hydrogen (+)-Tartrate Administration and Subsequent Withdrawal on Rat Liver Tryptophan Pyrrolase Activity and their Comparison with those of Morphine, Phenobarbitone and Ethanol

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(Received 25 November 1974)

1. Acute administration of nicotine hydrogen (+)-tartrate enhances the activity of rat liver tryptophan pyrrolase by a hormonal mechanism. 2. Chronic nicotine treatment inhibits, and subsequent withdrawal enhances, the pyrrolase activity. The inhibition during chronic treatment is not due to a defective apoenzyme synthesis nor a decreased cofactor availability. Regeneration of liver NADP⁺ *in vitro* and *in vivo* reverses the inhibition. 3. Chronic nicotine administration increases the liver NADPH concentration. 4. The above effects of nicotine resemble to a remarkable degree those previously shown for morphine, phenobarbitone and ethanol. All effects are compared, and their possible significance in relation to drug dependence is discussed.

The biochemical pathology of drug dependence is more likely to be advanced by examining what effects such lesion-producing drugs exert in common, rather than by trying to differentiate between them. A feature common to all drugs of dependence is their well-known ability to cause mood changes, with depression being the most prominent sign caused by their withdrawal. Although mood regulation appears complicated and may involve the participation of, and the possible interaction between, several biochemical processes, there is growing evidence implicating 5-hydroxytryptamine in mood disorders in drug dependence and other psychological conditions. It follows therefore that a study of the factors influencing the synthesis in the brain of this biogenic indoleamine may help us understand the mechanism(s) of the above disturbances of mood. One of these factors [discussed by Badawy & Evans (1974*b*)] is the activity of liver tryptophan pyrrolase (L-tryptophan-O₂ oxidoreductase, EC 1.13.11.11) which governs the quantitatively most important (kynurenine-nicotinic acid) pathway of tryptophan degradation, and which is inversely related to brain 5-hydroxytryptamine concentration (Curzon, 1969).

We have previously reported the effects of acute and chronic administration and subsequent withdrawal of ethanol, phenobarbitone and morphine on rat liver tryptophan pyrrolase activity (Badawy & Evans, 1973*a,b,c,d*, 1975). Whereas differences were found in the degrees with which these drugs activated the enzyme after acute administration, their chronic, as well as withdrawal, effects were remarkably similar, and involved inhibition and induction respectively. The present paper describes the results of similar

experiments with nicotine, and compares its effects with those of the above three drugs of dependence.

Materials and Methods

Chemicals

Actinomycin D was a gift from Merck, Sharp and Dohme, Hoddesdon, Herts., U.K. Alcohol dehydrogenase (from baker's yeast, 300 or 400 units/mg of protein; one unit converts 1 μ mol of ethanol into acetaldehyde/min at pH 8.8 and at 25°C), cortisol 21-acetate, cycloheximide, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (type XV, from baker's yeast, approx. 200–400 units/mg of protein; one unit oxidizes 1 μ mol of glucose 6-phosphate/min at pH 7.4 and at 25°C in the presence of NADP⁺), haemin (haematin hydrochloride), NAD⁺, NADP⁺ and L-tryptophan were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Ethanol (99.7–100%) and all other chemicals (from BDH Chemicals Ltd., Poole, Dorset, U.K.) were of AnalaR grade, except for dimethylformamide, D(-)-fructose, nicotine hydrogen (+)-tartrate, phenazine methosulphate (*N*-methylphenazonium methosulphate), phenolindo-2,6-dichlorophenol (dichlorophenol-indophenol), sodium pyruvate and sodium salicylate, which were of reagent grade.

Animals and injections

Male Wistar rats (150–225 g), maintained on cube diet MRC no. 41B, and water *ad libitum*, were killed between 12:00 h and 15:00 h by stunning and cervical dislocation. Rats used for chronic nicotine studies

(purchased from Carworth Europe, Anglia Laboratory Animals, Alconbury, Huntingdon, U.K.) each weighed $150\text{g} \pm 7\%$ at the start of the experiment. All chemicals were given intraperitoneally at various times before death as indicated in the Results section. For acute experiments, nicotine hydrogen (+)-tartrate, dissolved in 0.9% (w/v) NaCl, was administered in doses of up to 2mg/kg body wt. calculated as the free base. Cortisol acetate was dissolved in dimethylformamide and 0.1ml (2mg/100g body wt.) was injected. Dimethylformamide given alone (0.1ml/100g) was previously found (Badawy & Evans, 1973*d*) to give results identical with those of 0.9% NaCl. Thus the mean holoenzyme and total pyrrolase activities ($\pm$ S.E.M. of each group of four rats) were as follows: untreated rats, 1.6 ± 0.1 and 3.4 ± 0.2 ; 0.9% NaCl-treated rats, 1.8 ± 0.2 and 3.8 ± 0.4 ; dimethylformamide-treated rats, 1.7 ± 0.2 and 3.7 ± 0.4 . Actinomycin D (0.7mg/kg), cycloheximide (50mg/kg), fructose (400mg/kg), phenazine methosulphate (10mg/kg), sodium pyruvate (200mg/kg) and sodium salicylate (400mg/kg) were dissolved in 0.9% NaCl; each rat received 0.1–0.2ml/100g body wt. Tryptophan (200mg or 500mg) was dissolved in the minimum amount of 2M-NaOH and diluted to 10ml with 0.9% NaCl after the pH had been adjusted to 7.3 with 1M-HCl; each rat received 1ml/100g body wt. Ethanol (5ml/kg) was given as a 25% (v/v) solution in 0.9% NaCl; each rat received 2ml/100g body wt.

Chronic administration and withdrawal of nicotine

Rats were given a nicotine hydrogen (+)-tartrate solution as the sole source of drinking water *ad libitum*. A solution containing 3.08mg (1mg free base) per 100ml of water was provided freshly every morning. For withdrawal, the nicotine solution was replaced with drinking water. Body weight and fluid intake were recorded daily through the entire experimental period.

Preparation of homogenates

The liver was removed within 10s of the death of the animal, and was homogenized for 1min at 1100rev./min in 6.5vol. of 140mM-KCl–2.5mM-NaOH at 0°C in a glass homogenizer with a loose-fitting Teflon pestle. The homogenates were incubated within 3–6min of preparation.

Determination of liver tryptophan pyrrolase activity

The activity of the enzyme was determined in liver homogenates by measuring the formation of kynurenine from L-tryptophan (Feigelson & Greengard, 1961) in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added haematin. The apoenzyme activity was calculated by dif-

ference. Samples (15ml) of the homogenates were added to a solution containing 5ml of 0.03M-L-tryptophan, 15ml of 0.2M-sodium phosphate buffer, pH7.0, and 25ml of water at 0°C. Where necessary, haematin hydrochloride was dissolved in 0.1M-NaOH and 0.1ml was included in the overall mixture to give a final concentration ($2\mu\text{M}$) that was found optimum for enzyme activation (Badawy & Evans, 1974*a*). Higher concentrations of haematin (up to $8\mu\text{M}$) were also used in some experiments. Samples (3ml) of the assay mixture were incubated (for 90min or less) with shaking at 37°C in stoppered 25ml conical flasks in an atmosphere of O_2 . The reaction was stopped at 0, 15, 30, 45, 60 and 90min by the addition of 2ml of 0.9M-trichloroacetic acid; the flasks and contents were shaken for a further 2min and then filtered on Whatman no. 1 filter paper. To a measured portion of the filtrate (2.5ml) was added 1.5ml of 0.6M-NaOH and the kynurenine present was determined by measuring the E_{365} with a Unicam SP.500 spectrophotometer and by using $\epsilon = 4540 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. A lag phase persisted for the first 30–45min with the basal enzyme and the enzyme induced by cortisol. The enzyme activated by tryptophan, salicylate or ethanol had the lag phase either abolished or shortened to 15min. Tryptophan pyrrolase activity was calculated from the increase in the E_{365} with time during the linear phase. For an extinction range (at 365nm) of 0.1–1.0, the line representing the linear phase exactly covered points at three to four consecutive time-intervals. The validity of this visual method was mathematically assessed in a number of experiments, and was found satisfactory (r not less than 0.97). In the experiments *in vitro*, NAD^+ , NADP^+ and nicotine hydrogen (+)-tartrate were dissolved in the sodium phosphate buffer, adjusted to pH7.0 if necessary and added immediately before incubation.

Determination of liver nicotinamide-adenine dinucleotides

The concentrations of NAD^+ , NADH , NADP^+ and NADPH were determined directly in pieces (0.5g each) of livers of normal rats and of rats chronically treated with nicotine for 10 days. Both untreated and treated rats had a body-weight range of 220–232g. The extraction of NAD^+ and NADP^+ by ice-cold 2M- HClO_4 and that of NADH and NADPH by hot 0.1M-NaOH was done by the method of Slater & Sawyer (1962). Determination of all four nucleotides in liver extracts was performed as described by Slater *et al.* (1964), with two differences. A Unicam SP.500 spectrophotometer (at $15 \pm 0.5^\circ\text{C}$) was used, and a minor modification was adopted (T. F. Slater, personal communication) in which a 2mg/ml solution replaced the original 1mg/ml solution of phenazine methosulphate.

Results

Acute effects of nicotine on rat liver tryptophan pyrrolase activity

The addition of nicotine hydrogen (+)-tartrate (0.1–2mM) to normal rat liver homogenates *in vitro* did not significantly alter the basal pyrrolase activity, whether the assay mixture lacked (holoenzyme activity) or contained (total enzyme activity) added haematin.

The enzyme activity was increased when the nicotine salt (2mg of base/kg body wt.) was intraperitoneally injected (Fig. 1). Both holoenzyme and total

pyrrolase activities rose significantly ($P=0.05$) at 2h, reached a maximum 2.5-fold increase 1h later and finally fell to normal at 6h after the injection. The ratio of holoenzyme/apoenzyme activities (which is indicative of the degree of haem saturation of the apoenzyme) never exceeded the value (0.64) exhibited by the basal enzyme.

The effects of pretreatment of rats with actinomycin D or cycloheximide on the enhancement of the pyrrolase activity at 3h after nicotine administration are shown in Table 1. Both actinomycin D and cycloheximide prevented the nicotine-induced rise in the holoenzyme and total pyrrolase activities.

The results in Table 2 show the effects on holo-tryptophan pyrrolase activity of various combined injections of tryptophan, cortisol and nicotine. The combined injection of either tryptophan plus cortisol or tryptophan plus nicotine gave additive effects, in comparison with the sums of the corresponding separate ones, whereas that of cortisol plus nicotine did not. A broadly similar pattern of results was also obtained with the total pyrrolase activity.

The administration of nicotine at a dose (0.1 mg of base/kg body wt.) equivalent to that inhaled by man from a cigarette at the rate of four puffs/min for 7 min [with an average nicotine content of 0.25 mg of base/puff; Armitage (1965)] did not alter the pyrrolase activities at 3h. The mean control (0.9% NaCl) values for the holoenzyme and total enzyme activities (in μmol of kynurenine formed/h per g wet wt. of liver, $\pm$ S.E.M. of four rats) were 1.7 ± 0.1 and 4.8 ± 0.1 respectively. The corresponding values for the 0.1 mg/kg dose of nicotine were 1.9 ± 0.1 and 4.7 ± 0.4 respectively.

Chronic and withdrawal effects of nicotine on rat liver tryptophan pyrrolase activity

Rats chronically treated with nicotine gained weight at the normal rate. Each nicotine-treated rat (four per cage) drank an average amount of nicotine (base) of

Fig. 1. Time-course of the effects of intraperitoneal administration of nicotine hydrogen (+)-tartrate on rat liver tryptophan pyrrolase activity

Nicotine hydrogen (+)-tartrate (2mg/kg, calculated as the free base) was injected at zero time. Each point represents the mean value for four rats. The enzyme activity was determined, as described in the Materials and Methods section, in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added haematin (2 μM). ●, Holoenzyme activity; ○, total enzyme activity.

Table 1. Effects of pretreatment of rats with actinomycin D and cycloheximide on the nicotine enhancement of liver tryptophan pyrrolase activities

Each rat received an intraperitoneal injection of either 0.9% (w/v) NaCl or nicotine hydrogen (+)-tartrate (2mg of base/kg body wt.) 3h before being killed. Actinomycin D (0.7mg/kg) or cycloheximide (50mg/kg) were injected 1h before 0.9% NaCl or nicotine. The enzyme activity was determined, as described in the Materials and Methods section, in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added haematin (2 μM). Values are means $\pm$ S.E.M. of each group of four rats.

Pretreatment	Kynurenine formed ($\mu\text{mol/h}$ per g wet wt. of liver)			
	0.9% NaCl-treated rats		Nicotine-treated rats	
	Holoenzyme activity	Total enzyme activity	Holoenzyme activity	Total enzyme activity
Nil	1.7 ± 0.1	4.8 ± 0.1	4.7 ± 0.6	11.3 ± 1.1
Actinomycin D	1.5 ± 0.2	5.1 ± 0.5	1.4 ± 0.1	3.0 ± 0.4
Cycloheximide	1.9 ± 0.0	2.8 ± 0.2	1.5 ± 0.1	2.4 ± 0.2

Table 2. *Effects of nicotine, cortisol and tryptophan alone and in various combinations on rat liver tryptophan pyrrolase activity*

Each rat received an intraperitoneal injection of either 0.9% NaCl, nicotine hydrogen (+)-tartrate (2mg of base/kg), cortisol acetate (20mg/kg) or tryptophan (500mg/kg body wt.) singly or in various combinations 3h before being killed. The enzyme activity was determined, as described in the Materials and Methods section, in the absence of added haematin (holoenzyme activity). Values are means for four rats per group, from each of which a value (1.7) for the 0.9% NaCl-treated controls has been subtracted. Combined and separate injections mean that each group of rats received two agents or one agent respectively, whereas sums indicate the added effects of two agents given on separate occasions.

Injection(s)	Holoenzyme activity (μ mol of kynurenine formed/h per g wet wt. of liver)	
	Separate injections or sums	Combined injections
Nicotine	3.0	—
Cortisol	6.9	—
Tryptophan	6.5	—
Tryptophan+nicotine	9.5	10.6
Tryptophan+cortisol	13.4	13.5
Cortisol+nicotine	9.9	6.2

1.00–1.25mg/kg per day for the first 3–4 days of treatment, then 1.50–1.75mg/kg per day for the remainder of the chronic experimental period. All nicotine-treated rats were in apparently good health, and none died during the course of treatment.

The effects of chronic nicotine administration and subsequent withdrawal on rat liver tryptophan pyrrolase activity are shown in Fig. 2. The results of three separate experiments are shown as Figs. 2(a), 2(b) and 2(c). In all three experiments, nicotine was administered in drinking water at a constant daily concentration of 3.08mg of the salt (1mg of free base) per 100ml of water as described in the Materials and Methods section. In all three experiments, the total pyrrolase activity (but not that of the holoenzyme) was inhibited, whereas in Fig. 2(c) the inhibition was temporarily reversed for a brief period (between 13 and 15 days of treatment). This phenomenon is difficult to explain; it could be due to a change in environmental conditions or alternatively it may be related to the tolerance and/or stimulant properties of nicotine, since a similar change occurred during the inhibition of the pyrrolase by chronic treatment with amphetamine, which is also a central-nervous-system stimulant (A. A.-B. Badawy & M. Evans, unpublished work).

In all three experiments with nicotine, the holoenzyme activity was not significantly altered. The

Fig. 2. *Effects of chronic nicotine hydrogen (+)-tartrate administration and subsequent withdrawal on rat liver tryptophan pyrrolase activity*

In all three experiments (a, b and c), the nicotine salt was freely administered in drinking water as a 3.08mg (1mg of free base) per 100ml solution, as described in the Materials and Methods section, which also includes details of the determination of the enzyme activity. In (a) and (b), withdrawal of nicotine was started at the times indicated by the arrows, but no withdrawal was performed in (c). The holoenzyme activity was determined in the absence, whereas the total activity was determined in the presence, of added haematin (2μ M). The apoenzyme activity was calculated by difference. ●, Holoenzyme activity during chronic treatment; ○, total enzyme activity during chronic treatment; ▲, holoenzyme activity during withdrawal; △, total enzyme activity during withdrawal. Each point represents the mean value for four rats.

total enzyme activity, on the other hand, was significantly decreased (by 54%, $P < 0.001$) after 1 day of nicotine drinking, and remained inhibited (by 44–55%) for 17 days (Fig. 2a). The apoenzyme activity, calculated by difference, was therefore decreased (by 61–79%) for the same length of time. When withdrawal was started after 8 days of chronic nicotine treatment (Fig. 2a), the total pyrrolase activity returned to normal after 1 day, then both holoenzyme and total enzyme activities rose to 2–2.5-fold 3 days

Table 3. *Effects of NAD⁺ and NADP⁺ in vitro on the inhibition of rat liver tryptophan pyrrolase activity by chronic nicotine administration*

Rats were chronically treated with nicotine as described in the Materials and Methods section, and were tested at 18–19 days. NAD⁺ or NADP⁺ were added to liver homogenates from normal and chronic nicotine-treated rats at a final concentration of 2 mM. The enzyme activity was determined, as described in the Materials and Methods section, in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added haematin (2 μ M). Values are means $\pm$ S.E.M. for four rats per group.

Addition	Kynurenine formed (μ mol/h per g wet wt. of liver)			
	Control rats		Nicotine-treated rats	
	Holoenzyme activity	Total enzyme activity	Holoenzyme activity	Total enzyme activity
Nil	1.4 $\pm$ 0.1	3.0 $\pm$ 0.3	1.9 $\pm$ 0.1	1.9 $\pm$ 0.2
NAD ⁺	1.6 $\pm$ 0.1	3.7 $\pm$ 0.4	3.1 $\pm$ 0.3	8.3 $\pm$ 0.6
Nil	1.8 $\pm$ 0.2	4.4 $\pm$ 0.2	1.9 $\pm$ 0.1	1.9 $\pm$ 0.2
NADP ⁺	1.6 $\pm$ 0.2	4.7 $\pm$ 0.6	2.8 $\pm$ 0.3	8.6 $\pm$ 0.8

Table 4. *Comparison of tryptophan pyrrolase activity in liver homogenates from normal and chronic nicotine-treated rats after various treatments in vivo*

Nicotine was administered chronically as described in the Materials and Methods section, and the rats were tested between 8 and 18 days of treatment. Each normal and chronic nicotine-treated rat received a single intraperitoneal injection of either 0.9% (w/v) NaCl, cortisol acetate (20 mg/kg), tryptophan (200 mg/kg), sodium salicylate (400 mg/kg), ethanol [5 ml/kg, as a 25% (v/v) solution in 0.9% NaCl], fructose (400 mg/kg) or sodium pyruvate (200 mg/kg body wt.) 4 h before being killed. Some other rats (last two treatments) received 1 h before being killed an injection of 0.9% NaCl or of phenazine methosulphate (10 mg/kg). The enzyme activity was determined, as described in the Materials and Methods section, in either the absence (holoenzyme activity) or the presence (total enzyme activity) of added haematin (2 μ M). Values are means $\pm$ S.E.M. of each group of four rats, except that the untreated (nil) groups had means for 30 and 25 rats for the normal and nicotine-treated animals respectively.

Treatment	Kynurenine formed (μ mol/h per g wet wt. of liver)			
	Normal rats		Nicotine-treated rats	
	Holoenzyme activity	Total enzyme activity	Holoenzyme activity	Total enzyme activity
Nil	1.7 $\pm$ 0.1	3.8 $\pm$ 0.2	1.5 $\pm$ 0.0	1.8 $\pm$ 0.1
0.9% NaCl	1.7 $\pm$ 0.2	4.6 $\pm$ 0.4	1.6 $\pm$ 0.1	2.9 $\pm$ 0.0
Cortisol	11.0 $\pm$ 0.6	26.9 $\pm$ 2.6	13.6 $\pm$ 1.6	29.9 $\pm$ 1.7
Tryptophan	6.2 $\pm$ 0.4	9.3 $\pm$ 0.8	2.0 $\pm$ 0.2	4.0 $\pm$ 0.3
Salicylate	5.4 $\pm$ 0.4	8.8 $\pm$ 0.9	3.4 $\pm$ 0.4	5.0 $\pm$ 0.6
Ethanol	6.3 $\pm$ 0.5	10.1 $\pm$ 0.9	2.5 $\pm$ 0.2	5.0 $\pm$ 0.5
Fructose	2.3 $\pm$ 0.1	4.7 $\pm$ 0.5	1.2 $\pm$ 0.1	2.7 $\pm$ 0.1
Pyruvate	2.4 $\pm$ 0.2	5.4 $\pm$ 0.4	1.9 $\pm$ 0.1	2.3 $\pm$ 0.2
0.9% NaCl	1.7 $\pm$ 0.1	4.5 $\pm$ 0.3	1.7 $\pm$ 0.2	2.7 $\pm$ 0.2
Phenazine methosulphate	1.7 $\pm$ 0.1	3.3 $\pm$ 0.2	2.3 $\pm$ 0.2	4.9 $\pm$ 0.5

after withdrawal, and finally fell to the basal values 7 days after withdrawal.

In the second experiment (Fig. 2b), the apoenzyme activity, calculated by difference, was more strongly inhibited (by 73–100%) from day 2 of nicotine treatment, but the inhibition of the total pyrrolase activity was less strong (30%) on day 29. Withdrawal of nicotine after 21 days of chronic treatment (Fig. 2b) produced similar increases in both holoenzyme and total enzyme activities (2.3–3.0-fold) to those in the previously described experiment (Fig. 2a).

In the third experiment (Fig. 2c), the inhibition of

the total enzyme activity (38%) was significant ($P < 0.05$) after 5 days, and persisted for 43 days, except for the period between 13 and 15 days of treatment, when it was reversed.

Tryptophan pyrrolase activities in liver homogenates from rats chronically treated with nicotine were compared with those from normal rats after various treatments *in vitro* and *in vivo*. The addition of excessive amounts of haematin (up to 8 μ M) *in vitro* did not reverse the inhibition of the total pyrrolase activity in nicotine-treated rats. The mean activities for four rats chronically treated with nicotine for 18–19 days

(in μmol of kynurenine formed/h per g wet wt. of liver, $\pm\text{S.E.M.}$) in the absence or presence of 2, 4, 6 and $8\mu\text{M}$ -haematin were 2.0 ± 0.1 , 2.0 ± 0.1 , 2.0 ± 0.2 , 2.1 ± 0.2 and 2.0 ± 0.2 respectively. Reversal of the inhibition of the total enzyme activity by chronic nicotine administration was, however, observed in the presence of NAD^+ or NADP^+ *in vitro* (Table 3). Neither nucleotide had any significant effect on the basal pyrrolase activities of normal rat liver homogenates.

Cortisol administration was equally effective in nicotine-treated and in normal rats in inducing the pyrrolase activities (Table 4). The activation of the enzyme by the administration of tryptophan, salicylate or ethanol was, however, strongly decreased by chronic nicotine treatment. The inhibition of the total pyrrolase activity by this drug treatment was reversed at 1 h after the injection of phenazine methosulphate but not at 4 h after the administration of fructose or sodium pyruvate (Table 4). The administration of phenazine methosulphate into rats chronically treated with nicotine also produced, within a few minutes, acute withdrawal symptoms. These included teeth chattering, irritability to handling, chewing, ptosis, jumping and tremors.

The effects of chronic nicotine administration (for 10 days) on the concentrations of liver nicotinamide adenine dinucleotides are shown in Table 5. No significant changes in $[\text{NAD}^+]$, $[\text{NADH}]$, their sum or ratio were observed. The $[\text{NADPH}]$ was increased by 30% ($P<0.001$), whereas the $[\text{NADP}^+]$ was not. Chronic nicotine administration also increased the sum of $[\text{NADPH}]+[\text{NADP}^+]$ by 26% ($P<0.001$) and the ratio of $[\text{NADPH}]/[\text{NADP}^+]$ by 25% ($P<0.02$).

Discussion

Acute effects of nicotine on rat liver tryptophan pyrrolase activity

The intraperitoneal administration of nicotine enhances the activity of rat liver tryptophan pyrrolase (Fig. 1). This effect resembles the cortisol induction but differs from the tryptophan activation of the enzyme; this suggests that nicotine may act by releasing glucocorticoids because, although it also releases catecholamines (Kershbaum *et al.*, 1963), the latter activate the pyrrolase by a substrate-type, tryptophan-mediated mechanism involving a sequence of interactions between cyclic AMP, adipose-tissue lipase, serum non-esterified fatty acids and serum free (non-protein-bound) tryptophan (Badawy & Evans, 1975). The resemblance of the nicotine enhancement of tryptophan pyrrolase activity to that by cortisol is suggested by the results of three comparative experiments. Thus the ratio of holoenzyme/apoenzyme activities of the nicotine-induced enzyme resembles

that of the basal (0.64) and the cortisol-induced (0.69) enzyme, all of which are lower than that of the tryptophan-activated enzyme (2.0) (see Table 4 for the cortisol and tryptophan values in control rats). The combined injection of either tryptophan plus nicotine or tryptophan plus cortisol gives an additive effect on the pyrrolase activity (Table 2), thus suggesting different mechanisms of action by tryptophan on the one hand and nicotine or cortisol on the other. No additive effect is observed (Table 2) after the combined injection of nicotine plus cortisol, thus suggesting that these two agents do not act differently. Finally, the nicotine enhancement of the pyrrolase activities is prevented by pretreatment of rats with either actinomycin D or cycloheximide (Table 1), both of which prevent the cortisol induction (Badawy & Smith, 1971). The tryptophan activation of the pyrrolase is blocked by cycloheximide but not by actinomycin D (Badawy & Smith, 1971).

Chronic and withdrawal effects of nicotine on rat liver tryptophan pyrrolase activity

Chronic nicotine administration inhibits the total activity of rat liver tryptophan pyrrolase (Fig. 2). Since the holoenzyme activity is not altered, it may be concluded that the above inhibition is specific to the apoenzyme. Withdrawal of nicotine produces a rise in both holoenzyme and total pyrrolase activities. This rise is not primarily cofactor-dependent, since the haem saturation of the apoenzyme resembles that observed with the basal enzyme and the enzyme induced by cortisol (the holoenzyme/apoenzyme ratio in all three cases is less than 1). It is possible that this withdrawal-induced increase in the pyrrolase activities is due to a glucocorticoid-mediated induction.

The inhibition of the total pyrrolase activity by chronic nicotine administration is not due to an interference with the synthesis, availability or utilization of the haem activator, since excess of haematin *in vitro* does not reverse the inhibition. The inhibition is also not due to a defective apoenzyme synthesis, because cortisol is equally effective in nicotine-treated and control rats in inducing the enzyme (Table 4). The activation of pre-existing apoenzyme by acute administration of tryptophan (Knox, 1966; Schimke, 1969), salicylate, which acts by displacing protein-bound tryptophan (Badawy & Smith, 1971, 1972), or ethanol (Badawy & Evans, 1973a,b, 1975) is strongly decreased in rats chronically treated with nicotine (Table 4), presumably because the apoenzyme there is already inactive (Fig. 2).

The exclusion by the above experiments of factors that might have been responsible for the pyrrolase inhibition by chronic nicotine administration leaves us with the question as to whether physiological inhibitors are involved. Known physiologically occurring pyrrolase inhibitors include glucose

Table 5. Comparison of nicotinamide-adenine dinucleotide concentrations in livers of normal and chronic nicotine-treated rats

The concentrations of NAD⁺, NADH, NADP⁺ and NADPH were determined in pieces of livers, as described in the Materials and Methods section, from both normal and chronic nicotine-treated (10 days) rats. Both groups of rats were of similar age, strain, sex (male) and body-weight range (220–232 g). Values are means $\pm$ S.E.M. of the numbers of rats in each group as indicated in parentheses. * $P < 0.001$; † $P < 0.02$.

Determination	Concentration (μ g/g wet wt. of liver) or ratio	
	Normal rats	Chronic nicotine-treated rats
[NAD ⁺]	416 $\pm$ 18 (6)	393 $\pm$ 19 (6)
[NADH]	229 $\pm$ 6 (5)	229 $\pm$ 7 (6)
[NAD ⁺]+[NADH]	645 $\pm$ 13 (5)	622 $\pm$ 21 (6)
[NAD ⁺]/[NADH] ratio	1.79 $\pm$ 0.1 (5)	1.69 $\pm$ 0.2 (6)
[NADP ⁺]	46 $\pm$ 1 (6)	49 $\pm$ 3 (6)
[NADPH]	188 $\pm$ 5 (5)	245 $\pm$ 8* (6)
[NADPH]+[NADP ⁺]	234 $\pm$ 4 (5)	294 $\pm$ 11* (6)
[NADPH]/[NADP ⁺]	4.09 $\pm$ 0.2 (5)	5.10 $\pm$ 0.2† (6)

(Wetterberg *et al.*, 1970; Yuwiler *et al.*, 1970; Green-gard & Dewey, 1973), NADH and NADPH (Cho-Chung & Pitot, 1967) and nicotinamide (Yamaguchi *et al.*, 1967). The inhibition by nicotinamide occurs in hypophysectomized or adrenalectomized rats, but not in intact rats, which show induction, whereas that by glucose treatment may involve a metabolite(s) but not the sugar itself. NADH and/or NADPH, which act by an allosteric mechanism (Cho-Chung & Pitot, 1967), have been implicated in the inhibitory action of chronic administration of ethanol, phenobarbitone and morphine (Badawy & Evans, 1973*a,b,c*, 1975). In order to test their possible involvement in the inhibition, liver nicotinamide-adenine dinucleotide concentrations were determined in rats chronically treated with nicotine (Table 5). The drug treatment significantly increased the [NADPH], the sum of [NADPH]+[NADP⁺] and the ratio [NADPH]/[NADP⁺]. The possible involvement of increased [NADPH] in the inhibition was then tested, and is suggested by the finding (Table 4) that phenazine methosulphate, an agent that rapidly reoxidizes liver NADPH and also NADH (Katz & Wals, 1970), reverses the inhibition, whereas the regenerators of liver NAD⁺, fructose or pyruvate (Slater, 1972), do not. NADPH involvement is also suggested by the ability of NADP⁺, added *in vitro*, to reverse the inhi-

Table 6. Comparison of the effects of ethanol, phenobarbitone, morphine and nicotine on rat liver tryptophan pyrrolase activity and nicotinamide-adenine dinucleotide concentrations

(+) $\rightarrow$ (++++), Arbitrary scale of increase; +, positive effect or presence; -, negative effect or absence; (-), decrease.

Test	Ethanol	Phenobarbitone	Morphine	Nicotine
Acute administration				
Pyrrolase increase	(++++)	(+)	(+)	(++)
Mechanism	Substrate	Cofactor	Substrate or cofactor	Hormonal
Chronic administration				
Pyrrolase inhibition	+	+	+	+
Cortisol induction	+	+	+	+
Tryptophan activation	-	-	-	-
Salicylate activation	-	-	-	-
Ethanol activation	-	-	-	-
Reversal of inhibition by:				
fructose	+	-	-	-
pyruvate	+	-	-	-
phenazine methosulphate	+	+	+	+
NAD ⁺ <i>in vitro</i>	+	+	+	+
NADP ⁺ <i>in vitro</i>	+	+	+	+
Withdrawal symptoms by				
phenazine methosulphate	+	+	+	+
[NAD ⁺]	-	(-)	-	-
[NADH]	(++)	(-)	-	-
[NADP ⁺]	-	-	-	-
[NADPH]	(++)	(++)	(++)	(++)
Withdrawal				
Pyrrolase increase	(++)	(++)	(++)	(++)

bition (Table 3). The finding that NAD^+ addition *in vitro* also reverses the inhibition (Table 3), whereas its regeneration by fructose or pyruvate administration does not (Table 4), may be explained by NAD^+ being converted *in vitro* into NADP^+ by utilizing endogenous substrates such as glutamate.

Comparison of the effects of ethanol, phenobarbitone, morphine and nicotine on rat liver tryptophan pyrrolase activity and nicotinamide-adenine dinucleotides

The above effects are summarized in Table 6, and show remarkable similarities. Some of these effects deserve comment. The production by phenazine methosulphate of withdrawal symptoms in rats chronically treated with any of these four drugs of dependence is, to our knowledge, the first example of one compound producing withdrawal from dependence on more than one drug, and suggests the possible existence of a common mechanism of dependence on drugs. Although phenazine methosulphate may possess other actions, its effects on tryptophan pyrrolase activity in chronic drug-treated rats and on liver nucleotides may be of interest in relation to this common mechanism of dependence. Tryptophan pyrrolase activity is inversely related to brain 5-hydroxytryptamine concentration (Curzon, 1969), and the short half-life of the former, 1.3h (Shore *et al.*, 1971), suggests that significant and relatively rapid changes in the latter may occur during chronic treatment and subsequent withdrawal of the above drugs. Alternatively, the ability of phenazine methosulphate to regenerate NAD^+ and NADP^+ in the liver suggests that a similar effect in the brain may cause depletion of the reduced nucleotides which are necessary for the maintenance of the hydroxylases involved in the synthesis of biogenic amines in the brain. These possibilities require examination before drawing any definite conclusions regarding the significance of the present findings in relation to drug dependence.

Only phenobarbitone decreases the liver $[\text{NAD}^+]$ and $[\text{NADH}]$ (Table 6). This effect cannot be explained, but the synthesis and/or degradation of adenine nucleotides may be affected by phenobarbitone, as has been reported (Seifert & Vacha, 1974) for pyrimidine nucleotides. The increased liver $[\text{NADH}]$ by chronic ethanol treatment may be due to the drug's own metabolism (for reviews see Hawkins & Kalant, 1972; Slater, 1972; Badawy & Evans, 1974b), whereas the accumulation of NADPH by phenobarbitone, morphine, nicotine (as well as by ethanol) cannot yet be explained. It is possible that these drugs may affect some enzymes of glucose metabolism utilizing the $\text{NADP}^+/\text{NADPH}$ couple, or they could simply act on the rate of glucose utilization. Glucose metabolism has been implicated in the pyrrolase inhibition by the sugar treatment (Wetter-

berg *et al.*, 1970; Greengard & Dewey, 1973), and it is interesting that chronic glucose administration (A. A.-B. Badawy & M. Evans, unpublished work) inhibits the apo-(tryptophan pyrrolase) activity and subsequent withdrawal causes an enhancement, thus resembling the above four drugs of dependence.

Support by the Medical Research Council is gratefully acknowledged. We thank Professor T. F. Slater for helpful discussions, Merck, Sharp and Dohme for a generous gift of actinomycin D, Mr. A. Dacey for animal maintenance and Mr. C. J. Morgan, Miss Jill C. Davis and Mr. C. G. Brook for technical assistance.

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