The Effect of the Chronic Administration of D-Amphetamine upon Circadian Changes in Amino Acids in the Pineal and Pituitary Glands of the Rat

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The formation of dansyl derivatives of amino acids, 5-hydroxyindoleacetic acid and histamine and their separation on polyamide plates provided a reliable and sensitive method for studying circadian changes in single pineal and pituitary glands of the rat. There appears to be no correlation between the circadian changes in concentrations of these substances in the pineal and pituitary glands.

Chronically administered D-amphetamine altered the circadian rhythms of 5-amino acids in the pituitary, including the putative transmitters taurine, glycine and glutamate; in the pineal gland only the rhythmical changes of lysine and 5-hydroxyindoleacetic acid were affected.

Introduction

Many of the cardinal symptoms resulting from chronic D-amphetamine abuse are similar to those seen in certain types of psychotic illness [1, 2]. Several investigators have studied the chronic effects of this drug on the behaviour and biochemistry of animals with a view to establishing an animal model for the psychoses [3–5]. While the results of the experimental investigations show promise of support for this model, no consideration seems to have been given to the effect of chronic amphetamine on rhythmical physiological changes. It is well established that the concentrations of neurotransmitters [6, 7], free amino acids [6, 8, 9], proteins and nucleic acids [10, 11] as well as adrenocortical hormones [7] show considerable fluctuations over a period of 24 hours. If amphetamine causes a change in the circadian rhythm of these substances, then such changes should be taken into account when interpreting the data obtained from studies in which drug and non-drug treated animals are compared.

The following investigation was undertaken to see what effects chronic amphetamine treatment would have on the free amino acid composition of the pineal and pituitary glands. It is already established that the rodent pineal gland, amines and metabolites shows distinct circadian rhythms [6]; blood corticosteroid levels also show rhythmic changes [7] presumably reflecting changing activity of the pituitary gland. It therefore seemed worthwhile to study changes in free amino acids in these tissues using the sensitive dansyl chloride microanalytical procedure [12].

Materials and Methods

Groups of 5 ♂ albino rats, initially weighing 100–110 g, were housed in Macralon cages and allowed free access to food and either tap water (control groups) or amphetamine in water at a concentration of 50, 100 and 200 mg/l for the first, second and third week of the experiment respectively. Some 200 mg/l of ascorbic acid was added to the drinking water to reduce the oxidation of the drug; the solutions were changed at least every second day. This method of giving D-amphetamine has been found to cause stereotyped behaviour and pronounced changes in brain amine metabolism during and after a 3 week period of administration [13]. The lighting conditions throughout the experiment were standard for the animal holding unit; the rats were exposed to light from 0700 to

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Table I. Times for the maximum and minimum changes in some free amino acids in the pineal and pituitary glands of the rat.

Amino acid		Pituitary Gland		Pineal Gland	
		Maximum	Minimum	Maximum	Minimum
Taurine	C	0800 *	0200	1400	2000
	E	2000	1400	0800	2000
Lysine	C	not detected		2000	0200
	E	not detected		0200	1400
Ornithine	C	1400	0200	0800	2000
	E	1400	0800	no apparent change	
Leucine	C	0200	0800, 1400, 2000 **	no apparent change	
	E	no apparent change		no apparent change	
Glutamine + Aspartate	C	1400	0200	0800	2000
	E	2000	0200	0800	2000
Glutamate + Aspartate	C	2000	0800	1400	2000
	E	1400	0200	1400	2000
Glycine	C	0200	1400	8000	0800
	E	0800	2000	2000	0800
Alanine	C	0200	2000	1400	2000
	E	0200	2000	1400	2000
Ethanolamine	C	0800, 1400, 2000	0200	2000	0200
	E	1400	0200	2000	0200
Proline	C	0800, 2000, 0200	1400	2000	0800
	E	1400	0800	2000	0800
Phenylalanine etc.	C	0200	2000	no apparent change	
	E	0200	1400	0800	2000
Tyrosine	C	1400	0800	0200, 0800, 1400	2000
	E	1400	0200	0200, 0800, 1400	2000
5-Hydroxyindole-acetic acid	C	0200	2000	0200	2000
	E	0200	1400	1400	0200
Histamine	C	0200	2000	0200	2000
	E	no apparent change		0800	2000

* Time in hours. Phenylalanine etc. = phenylalanine + isoleucine + histidine. C = control rats, E = amphetamine treated rats.

** The concentration of the substance was apparently unchanged when determinations were made at these times.

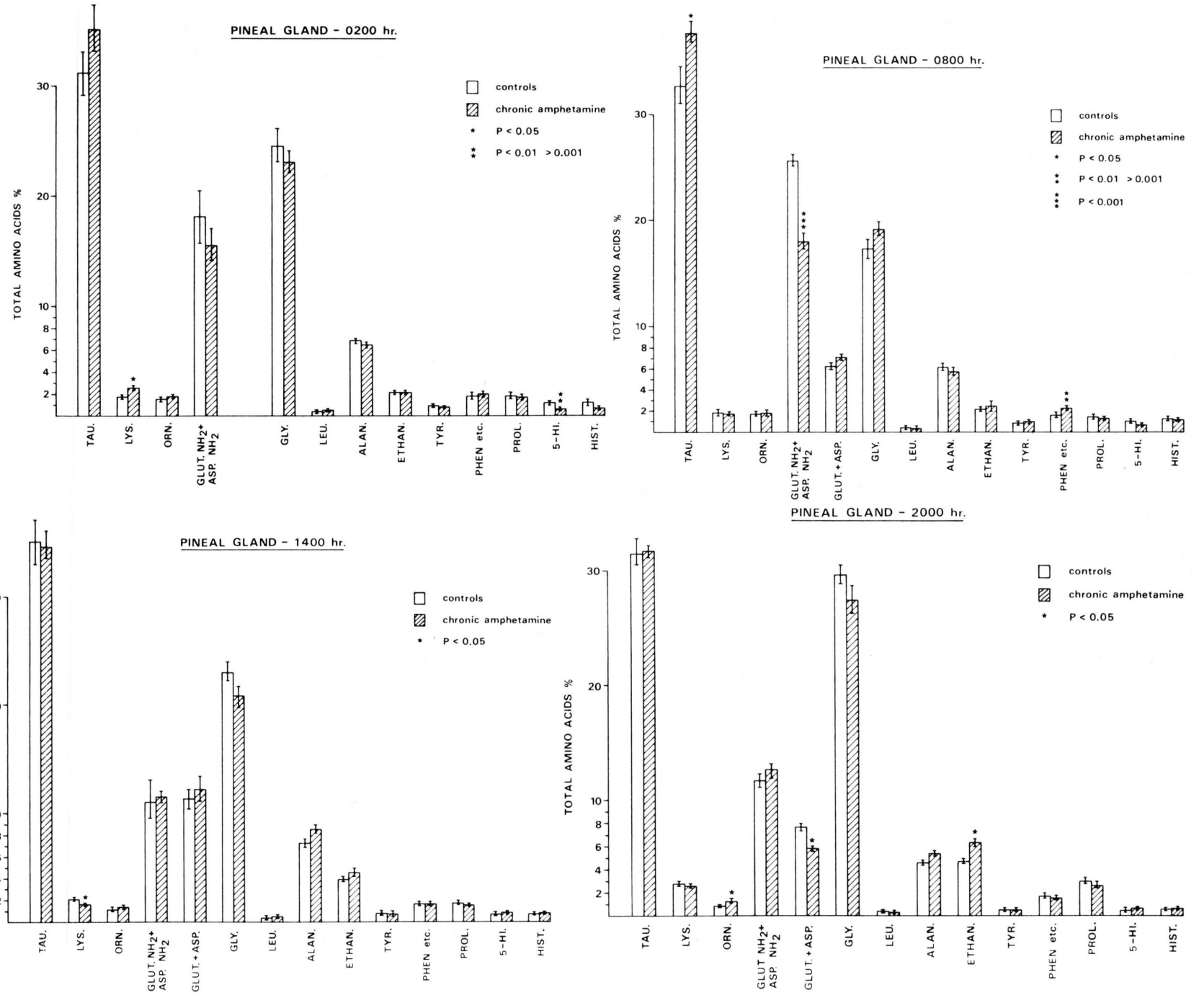
1800 hr daily. After 3 weeks, groups of 5 experimental and 5 control rats were killed by decapitation at 0200, 0800, 1400 and 2000 hr. The pineal and pituitary glands were rapidly removed and frozen on solid carbon dioxide. The whole pineal gland was weighed and homogenized in 20 μl of 0.05 M sodium bicarbonate solution (pH 10). Pieces of the pituitary gland weighing approximately 1 mg were removed by means of a stainless steel punch of the type described by Neuhoff[14], weighed and homogenized in 20 μl of the bicarbonate solution.

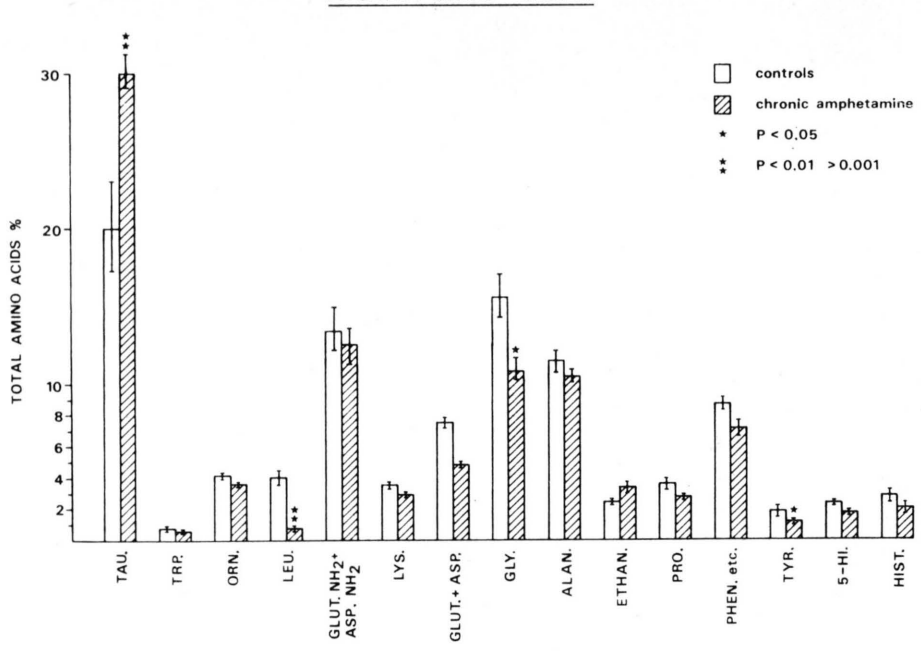
Preparation and separation of the dansylated amino acids and amines

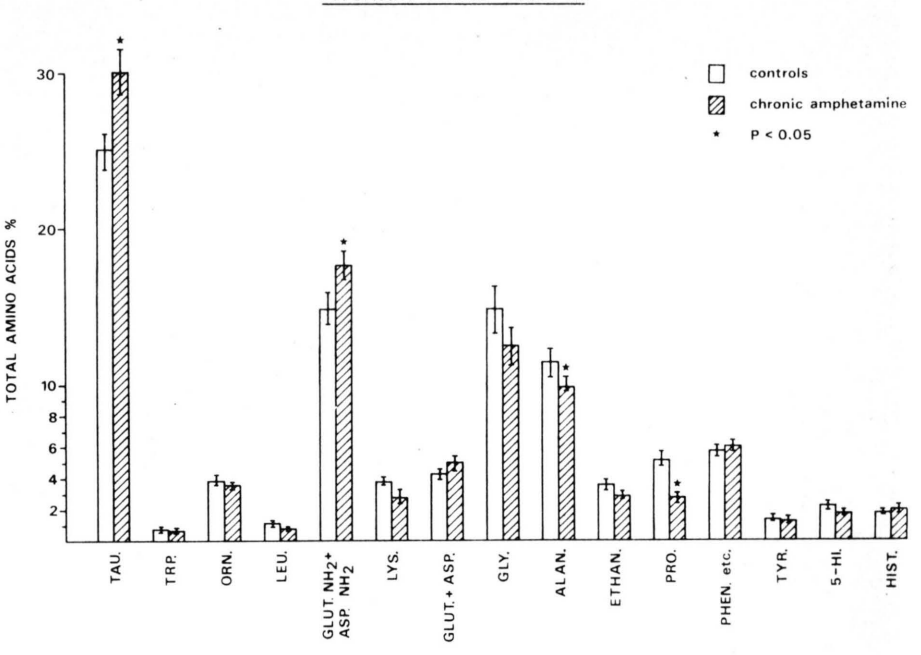
The homogenates were centrifuged at approximately 50,000 $\times g$ for 30 min and 20 μl of acetone added to the resulting supernatant to precipitate any low molecular weight proteins. The solution was again centrifuged and 4 μl samples of the supernatant removed for dansylation. 4 μl of [^{14}C]dansyl chloride solution in acetone (2.7 mg/ml; specific activity 49 mCi/mmol) was then added to the 4 μl of the supernatant and the mixture incubated at 37 °C for 30 min evaporated to dryness *in vacuo* and the residue dissolved in 4 μl of acetone : glacial acetic acid (3:2, v/v) solution. 0.2 – 0.5 μl aliquots of the dansylated products were applied to 3 cm × 3 cm polyamide plates and separated by 2 dimen-

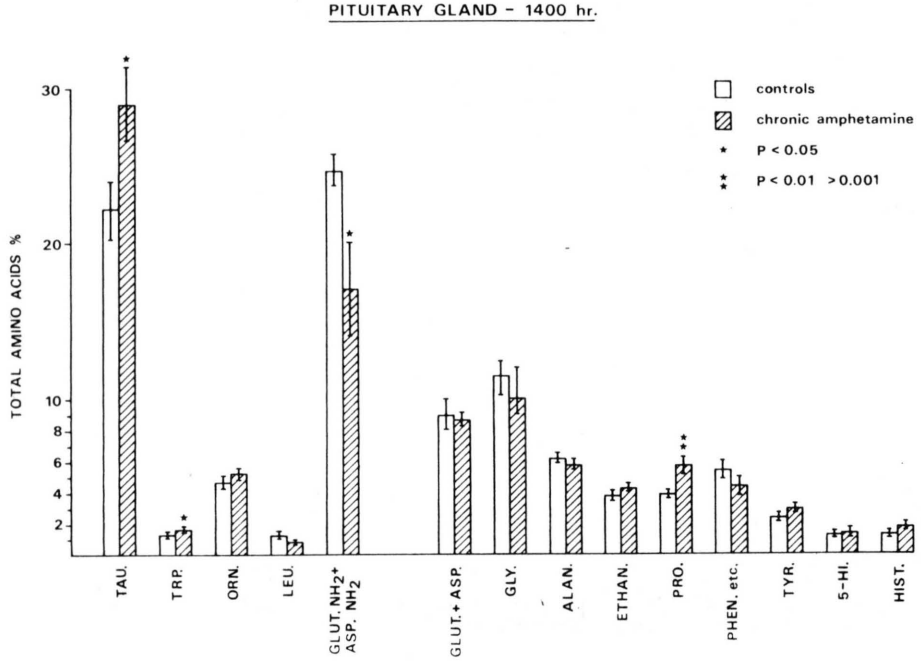
Fig. 1. Circadian changes (a – d) in free amino acids in the rat pineal gland. Results shown as the mean ± s.e.m. of the radioactivity (dpm) as a percentage of the total radioactivity of all the substances estimated.

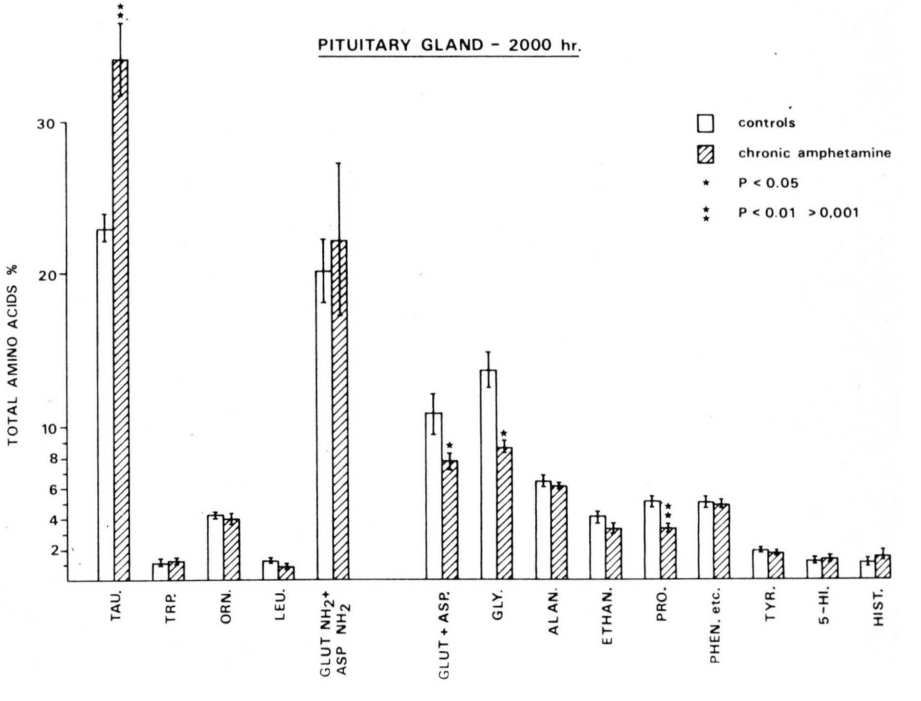
Tau = taurine, Lys = lysine, Orn = ornithine, Glut NH_2 + Asp NH_2 = glutamine + asparagine, Glut+Asp. = glutamate + aspartate, Gly = glycine, Leu = leucine, Alan = alanine, Ethan = ethanolamine, Tyr = tyrosine, Phen etc = phenylalanine+isoleucine+histidine, Pro = proline, 5-HI = 5-hydroxyindoleacetic acid, Hist = histamine.


PINEAL GLAND - 0200 hr.
controls
chronic amphetamine
* P < 0.05
** P < 0.01 >0.001
TOTAL AMINO ACIDS %
TAU.
LYS.
ORN.
GLUT. NH2+ ASP. NH2
GLY.
LEU.
ALAN.
ETHAN.
TYR.
PHEN etc.
PROL.
5-HI.
HIST.
PINEAL GLAND - 0800 hr.
controls
chronic amphetamine
* P < 0.05
** P < 0.01 >0.001
*** P < 0.001
TOTAL AMINO ACIDS %
TAU.
LYS.
ORN.
GLUT. NH2+ ASP. NH2
GLUT. + ASP.
GLY.
LEU.
ALAN.
ETHAN.
TYR.
PHEN etc.
PROL.
5-HI.
HIST.
PINEAL GLAND - 1400 hr.
controls
chronic amphetamine
* P < 0.05
TOTAL AMINO ACIDS %
TAU.
LYS.
ORN.
GLUT. NH2+ ASP. NH2
GLUT. + ASP.
GLY.
LEU.
ALAN.
ETHAN.
TYR.
PHEN etc.
PROL.
5-HI.
HIST.
PINEAL GLAND - 2000 hr.
controls
chronic amphetamine
* P < 0.05
TOTAL AMINO ACIDS %
TAU.
LYS.
ORN.
GLUT NH2+ ASP NH2
GLUT. + ASP.
GLY.
LEU.
ALAN.
ETHAN.
TYR.
PHEN etc.
PROL.
5-HI.
HIST.


PITUITARY GLAND - 0200 hr.
TOTAL AMINO ACIDS %
controls
chronic amphetamine
* P < 0.05
** P < 0.01 >0.001
TAU.
TRP.
ORN.
LEU.
GLUT. NH2+ ASP. NH2
LYS.
GLUT.+ ASP.
GLY.
ALAN.
ETHAN.
PRO.
PHEN. etc.
TYR.
5-HI.
HIST.


PITUITARY GLAND - 0800 hr.
TOTAL AMINO ACIDS %
controls
chronic amphetamine
* P < 0.05
TAU.
TRP.
ORN.
LEU.
GLUT. NH2+ ASP. NH2
LYS.
GLUT.+ ASP.
GLY.
ALAN.
ETHAN.
PRO.
PHEN. etc.
TYR.
5-HI.
HIST.


PITUITARY GLAND - 1400 hr.
TOTAL AMINO ACIDS %
controls
chronic amphetamine
* P < 0.05
** P < 0.01 >0.001
TAU.
TRP.
ORN.
LEU.
GLUT. NH2+ ASP. NH2
GLUT.+ ASP.
GLY.
ALAN.
ETHAN.
PRO.
PHEN. etc.
TYR.
5-HI.
HIST.


PITUITARY GLAND - 2000 hr.
TOTAL AMINO ACIDS %
controls
chronic amphetamine
* P < 0.05
** P < 0.01 >0,001
TAU.
TRP.
ORN.
LEU.
GLUT NH2+ ASP NH2
GLUT + ASP.
GLY.
ALAN.
ETHAN.
PRO.
PHEN. etc.
TYR.
5-HI.
HIST.

sional chromatography using formic acid : water (3 : 100, v/v) in the first dimension and benzene : acetic acid (9 : 1, v/v) in the second dimension. A mixture of approximately 30 dansyl derivates could be separated in this way. After carefully drying the plates, the products were visualized under ultraviolet light. The perimeters of the spots were marked with a soft pencil; the spots were removed by means of a microknife and transferred to a liquid scintillation vial containing 10 ml of scintillator (PPO, POPOP, toluene). The radioactive counts of the samples were determined using a Packard liquid scintillation spectrometer.

The full details of the method, together with the precautions which must be applied in undertaking such a procedure, have been described elsewhere [12, 14]. A total of 14 dansylated substances was determined for each microchromatogram; in most cases triplicated estimations were made on each tissue. The radioactivity (as dpm) of each dansylated product was expressed as a percentage of the total radioactivity of all substances determined.

Reagents

Analytical grade reagents were used whenever possible. The polyamide plates were purchased from Schleicher and Schüll (TLC Ready-Plastic Sheets, F1700, Micro-Polyamide). The [^{14}C]dansyl chloride solution was obtained from Schwartz/Mann, Orangeburg, New York.

Results

The results of this investigation are summarized in Figs 1 and 2. It can be seen that chronically administered D-amphetamine affects the relative concentrations of several amino acids in the pituitary gland; the effects of the drug on pineal amino acids are less marked. There is also evidence that the rhythmicity of the circadian changes is affected by D-amphetamine. This is shown more clearly in Table I, where the times at which amino acid concentrations were maximal and minimal are summarized. It can also be seen that the times for the maximum and minimum changes for several of the amino acids differ between the two glands. D-amphetamine was found to alter the time for both the maxima and minima of 4 of the substances quantified in this study in the pituitary (taurine, glutamate, glycine and proline); only the lysine and 5-hydroxyindoleacetic acid rhythms were affected in the pineal.

It was of interest to find that γ-aminobutyric acid could not be detected unequivocally in the rat pineal and pituitary glands even though we have shown that this amino acid is present in a relatively high concentration in the hypothalamus of this species [15].

Discussion

Two points emerge clearly from this study. First, the changes induced by the chronic administration of D-amphetamine were more pronounced in the pituitary gland; of the 14 parameters determined, amphetamine was found to significantly affect 9 of them, whereas only 7 parameters were affected in the pineal. Second, there is evidence that the circadian rhythms of several amino acids and of 5-hydroxyindoleacetic acid are altered by the drug. Observation of the gross behaviour of both groups of rats immediately before they were killed showed that the drug treated aminals were grossly hyperactive, even during the period of daylight. It is possible that some of the behavioural anormalities shown by the drug-treated animals may be attributed to the disturbed circadian rhythms. The non-drug treated rats showed maximal activity during the hours of darkness and were considerably less active during the day. Such changes in the free amino acids of the untreated rats may reflect an altered synthesis, as it has been shown that protein synthesis in the pineal glands is reduced during periods when animals are exposed to light [11, 16]. However, it is also possible that the changes in taurine, glycine, glutamate and alanine, and in 5-hydroxyindoleacetic acid reflect a fluctuation in neurotransmitter function.

The inhibitory amino acid γ-aminobutyric acid could not be unequivocally detected in either the pineal or pituitary glands even though the relative concentrations of its precursors glutamate and glutamine, were very high in both of these tissues. This implies that not only is γ-aminobutyric acid unlikely to have a transmitter role in these tissues but also that "GABA-shunt" activity, which is high in the surrounding brain tissue [17] is very low. In contrast, the concentration of this amino acid is particularly

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Fig. 2. Circadian changes (a—d) in free amino acids in the rat pituitary gland. Details are as given in legend to **Fig. 1.**

high in the hypothalamus [15], an area of the brain which is functionally closely connected with the pituitary. It is possible that the neurotransmitter role of γ-aminobutyric acid in the pineal and pituitary glands is replaced by that of taurine. This amino acid has been proposed as a neurotransmitter in other regions of the mammalian brain [18]; neurophysiological studies have also shown that its ability to depress neuronal firing when applied microiontophoretically to cells in the cortex, spinal cord, and medulla suggests that it has an action which is quite distinct from that of γ-aminobutyric acid [19].

Using the more conventional quantitative techniques for the estimation of taurine, Guidotti *et al.* [20] also showed that the concentration of this amino acid was particularly high in the pituitary and pineal glands of the cat. More recently, Neuhoff and Tonge [21] presented evidence to suggest that this amino acid may have a role in the regulation of hormone secretion in the mouse pituitary gland. The possibility therefore arises that the complex behavioural and physiological changes which occur following the chronic administration of amphetamine may be partly attributable to an altered hormonal control mechanism.

The changes occurring in the relative concentrations of the free amino acids during the 24 hour period were not nearly as marked as other investigations have reported for the brain; the levels of amino acids in the brain have been reported to be 6 – 14 fold greater during the period of maximum activity compared with that of minimum activity [22, 23]. Leonard and Shallice [24] showed that there was a 100% change in the concentration of brain biogenic amines, their precursor amino acids and γ-aminobutyric acid during the period from 09.30 to 21.30 hr. Clearly, circadian fluctuations in amines and amino acids must be carefully taken into account whenever detailed neurochemical studies of drug action are being undertaken, especially as it has been shown that hormonal fluctuations affect brain monoamine metabolism [25] and alter the neurochemical responses to psychotropic drugs [26].

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