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BIOCHEMICAL STUDIES OF CEREBRAL SUB-FRACTIONS AFTER CHRONIC ADMINISTRATION OF PYRIDAZINE (*N*-MORPHOLINE 3-ETHYLAMINE 4-PHENYL 6-PYRIDAZINE HYDROCHLORIDE, AG 620)

M-R. ORNELLAS

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), De Crespigny Park, London S.E.5

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Abstract—The effects of chronic administration of pyridazine (*N*-morpholine 3-ethylamine 4-phenyl 6-pyridazine hydrochloride) on total protein, RNA content and ATPase and AChE activities of subcellular fractions of rat brain were determined. The subcellular fractions were prepared by separation on a discontinuous sucrose gradient after removal of nuclei and debris. It was found that the drug increased the total protein in the fractions corresponding to the microsomes and synaptosomes but no increase of RNA was observed in the same fractions. The drug caused inhibition of Na^+,K^+-ATPase which was greatest in the microsomal fraction and of AChE, greatest in the fraction corresponding to the synaptosomes.

IN A PREVIOUS publication[1] it was reported that pyridazine (*N*-morpholine 3-ethyl amine 4-phenyl 6-pyridazine hydrochloride) which acts as a tranquilizer when administered to the rat in a dose of 5 mg/kg acts as a convulsant if the dose is increased to 10–20 mg/kg.

Chronic administration of the drug in tranquillizing doses resulted in an increase of the body weight and a slight increase of the weight and total protein content of the forebrain associated with a decrease of Na^+, K^+-ATPase and AChE* activities.

In order to study the possibility that in addition to ATPase and AChE activities changes in metabolism of RNA and protein may result from treatment with drug, determinations of RNA, protein and enzyme activity have been made on subcellular components separated on one density-gradient.

ATPase and AChE activities are found distributed mainly in the membrane fractions of the microsomes and the nerve endings.[2,3] RNA is largely found, in association with protein, in the ribonucleoprotein granules of the rough membranes of the endoplasmic reticulum which is the major component of crude microsomal fractions.[4,6–8]

The complex structure of the brain imposes some technical difficulties in such a project but the object was not to isolate pure uncontaminated subcellular fractions but to obtain subfractions enriched in one or another of the morphological constituents.

* Abbreviations used: Na^+, K^+-ATPase, Na^+ and K^+ activated adenosine triphosphatase. AChE, Acetylcholinesterase.

As a result of preliminary study, a discontinuous sucrose gradient, where the fractions were well delineated, was adopted. Electron microscopic criteria for the identity of the fractions were not used; the fractions were identified by density characteristics.[5,9,10]

It is not unreasonable to assume that the drug employed (pyridazine) may induce changes in the density of some of the subcellular constituents with repeated dosage and this would be revealed by changed pattern of material on the gradient. The gradient allows comparison of specific activities in the subcellular fractions under comparable conditions.

EXPERIMENTAL

Chronic drug administration. Wistar male or female rats (weighing 200–250 g) were injected intraperitoneally during 15 days (5/7) with 5 mg/kg/day of the drug and the control group were similarly treated with the same volume of saline solution used as solvent. After treatment, the rats were struck on the neck, the forebrains were weighed and placed in ice-cold 0·32 M sucrose.

Primary fractionation. All operations were conducted at 0–4° and all sucrose solutions contained 5 mM tris-HCl buffer, pH 7·4. The initial 10 per cent dispersion was prepared by homogenization in a Teflon-pestle homogeniser (clearance of 150–250 μm making five passes at 1000 rev/min) and nuclei and tissue debris were removed by centrifugation at 600 *g* for 10 min. The pellet was washed twice by resuspension in 0·32 M sucrose and recentrifuged. The supernatants from the three centrifugations were pooled and centrifuged at 40,000 rev/min for 60 min in a Spinco Model L preparative ultracentrifuge.

Density gradient separation. The resultant pellet was resuspended in 10 ml of 0·32 M sucrose and 4 ml of the suspension, containing about 71·4 mg of tissue equivalent/ml, was layered on the top of a discontinuous sucrose gradient in Lustroid tubes of the SW25 head of the Spinco Model L preparative ultracentrifuge. The gradient, set up 5 hr before use, consisted of 10 ml of 1·2 M sucrose; 8 ml of 0·8 M sucrose and 8 ml of 0·6 M sucrose. After centrifugation at 25,000 rev/min for 2 hr, the top and intermediate bands were separated at 0° with calibrated pipette of suitable volume (previously calculated). Each fraction was examined under the light microscope for contamination by large elements (nuclei and unbroken cells) and the tentative identification of mitochondria. Fractions A–D appeared membraneous or vesicular increasing in size and density. Some small mitochondria were seen in fraction E, whilst fraction F consisted mainly of mitochondria with a very few nuclei.

ANALYSIS OF FRACTIONS

Proteins. Total protein was determined by the method of Miller.[11]

RNA. Each fraction of the gradient was treated by the method of Schmidt and Tannhauser[12] with some modifications. A small quantity of glass powder was placed in the bottom of each tube to fix the precipitate and to avoid the formation of a pellicle by triturating with a glass rod. TCA was added to the diluted fractions to give a final concentration of 6% (v/v). The mixtures were allowed to stand at 0° for 30 min and then centrifuged at 3500 rev/min for 20 min. The supernatants were discarded and the residues were washed twice with 6% TCA and then extracted success-

ively with ethanol–ether (1:1), chloroform–methanol (2:1), chloroform–methanol–0·2 N HCl (2:2:1) and ether. For estimation of phospholipids the first step (ethanol saturated with sodium acetate) recommended by Steele *et al.*,[13] cannot be used as the sodium acetate is insoluble in ether. Great importance was given to the first precipitation and to the time and speed of centrifugation. All manipulations were carried out at 0–4° to the stage of chloroform–methanol extraction. The dry fractions were wet by addition of one drop of glass distilled water and suspended in N NaOH, stirred and allowed to stand for 1 hr at room temperature. The procedures for the determination of RNA followed that described by Scott *et al.*[14] with modifications.

As the amount of RNA in the fractions was low in these experiments, the RNA was determined by the two wave-length method described by Tsanev and Markov[15] and Balázs and Cocks.[16] The amount of RNA-P was calculated by the formula given by Tsanev and Markov.[15]

Inorganic phosphate. This was determined by the method of Berenblum and Chain[17] with modifications described by Martin and Doty[18] and Linberg and Ernstner.[19]

Adenosinetriphosphatase activity. The determination was performed according to the method of Schwartz *et al.*[2]

Acetylcholinesterase activity. This was determined by the colorimetric determination of Ellman *et al.*[20]

RESULTS

After centrifugation the material was distributed throughout the gradient in six fractions (Fig. 1). Fraction A was the clear uppermost zone corresponding to the initial dispersion. Fraction B corresponded to the myelin which always appeared there whatever the molarity of the lower layers. Fraction C appeared only when the microsomal fraction was present in the pellet and was absent when only mitochondrial and synaptosomal fractions were layered on the top of a similar gradient. Fractions D and E were closely related and occurred in same position in the absence of the microsomal fraction. Fraction D was diffuse and not as concentrated as was fraction E. Fraction F consisted of the sediment in the bottom of the tubes. No material appeared intermediate between fractions E and F.

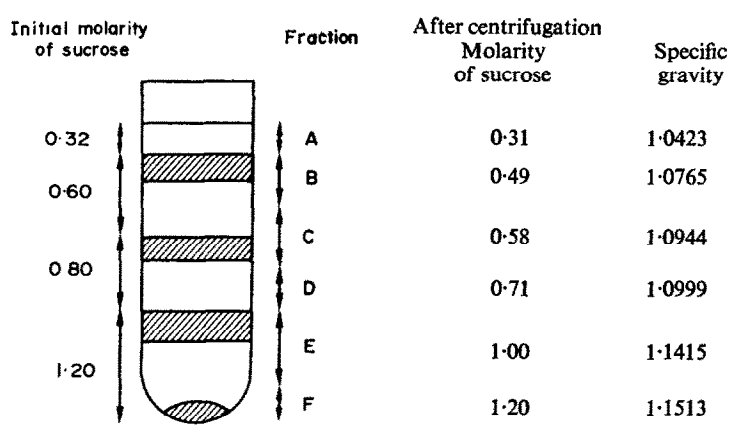

FIG. 1. Distribution of subcellular constituents after centrifugation at 25,000 rev/min for 2 hr.

Morphological identification of the fractions (Table 2) is that of Gray and Whittaker,[5] Potter and Axelrod[21] and De Robertis.[8] From the determination of RNA the presence of smooth membranes in fraction C was indicated. The rough membranes are localised in fraction E and the free ribosomes in the pellet with the mitochondria (fraction F). The densities of fractions shown in Table 1 are the corresponding densities of sucrose solutions after centrifugation calculated by refractive index.

The increase in the body weight (Table 1) was not reflected by any change in the forebrain weight. The previous observation that the drug induced an increase in total protein was confirmed.

TABLE 1. BODY WEIGHT AND FOREBRAIN WEIGHT AFTER TREATMENT BY PYRIDAZINE

Body weight (% increase over 3 weeks)		Forebrain weight (g)	
Control	Treated	Control	Treated
16·69 ± 3·58 (10)	23·90 ± 5·59 (10)	1·315 ± 0·057 (10)	1·334 ± 0·053 (10)

Values are the results of 10 animals (mean ± S.D.).

TABLE 2. DISTRIBUTION OF PROTEINS IN SUBCELLULAR FRACTIONS PREPARED BY DENSITY GRADIENT CENTRIFUGATION

Fraction	Particle density	Morphological identification	Proteins (mg/g of tissue)	
			Control	Treated
		Homogenate	124·69 ± 7·50	129·74 ± 5·98
Primary fractions:				
1		Nuclei and debris	11·11 ± 0·62	11·78 ± 0·62
2		Post-nuclear particulate fraction	115·35 ± 3·13	118·40 ± 5·04
3		Supernatant	30·50 ± 1·30	31·40 ± 1·41
Subfractions from 2:				
A	1·0423	No organised structures	2·45 ± 0·14	2·58 ± 0·15
B	1·0765	Myelin	5·74 ± 0·18	5·93 ± 0·31
C	1·0944	Smooth membranes	14·03 ± 1·07	16·10 ± 0·80
D	1·0999	Synaptosomes (light membranes)	9·07 ± 0·54	8·71 ± 0·42
E	1·1415	Synaptosomes and rough membranes	26·03 ± 1·20	28·29 ± 0·54
F	1·1513	Mitochondria and free ribosomes	20·67 ± 0·66	21·90 ± 0·57
Subfractions from 2: resuspended			76·90 ± 5·54	78·90 ± 5·54
Recovered %			102·70	102·60

Values are the results of 10 experiments (mean ± S.D.).

As shown in Table 2 the increase of protein was relatively high in the smooth microsomal fraction (fraction C) and in fraction E corresponding to the synaptosomes and rough membranes, although slight increases were found in almost all fractions. However, no correlated increase of RNA or phospholipid could be detected in the same fractions.

Chemical analysis revealed a relatively high concentration of RNA in the fraction F which contained mitochondria and free ribosomes and a low concentration of RNA in fraction C. These results are in agreement with those of Hanzon and Toschi[22] who separated the postmitochondrial supernatant on density gradients.

As shown in Table 3 the only increase observed as a result of treatment with the drug was a slight increase of the RNA of fraction F.

TABLE 3. DISTRIBUTION OF RNA-P AND PL-P IN THE FRACTIONS PREPARED BY DENSITY GRADIENT SEPARATION

	RNA-P (μatoms P/g of tissue)		PL-P (μatoms P/g of tissue)	
Fraction	Control	Treated	Control	Treated
A	0·098 ± 0·005	0·101 ± 0·014	1·27 ± 0·20	1·52 ± 0·29
B	0·188 ± 0·028	0·203 ± 0·023	5·94 ± 0·77	6·10 ± 0·45
C	0·382 ± 0·028	0·400 ± 0·028	19·85 ± 2·19	20·85 ± 1·39
D	0·284 ± 0·048	0·288 ± 0·073	11·77 ± 0·92	10·39 ± 0·89
E	1·231 ± 0·061	1·281 ± 0·084	22·09 ± 2·05	22·76 ± 1·00
F	1·092 ± 0·089	1·294 ± 0·095	12·88 ± 0·59	13·62 ± 0·60
Primary fractions from 2 resuspended	3·143 ± 0·340	3·333 ± 0·325	64·76 ± 2·54	67·49 ± 3·43

Values are the results of six experiments (mean ± S.D.).

TABLE 4. ADENOSINETRIPHOSPHATASE (ATPASE) ACTIVITY IN FRACTIONS PREPARED BY DENSITY GRADIENT CENTRIFUGATION

	(μmoles inorganic P liberated ATPase mg of protein/hr)					
	Control			Treated		
Fraction	With Na^+	Without Na^+	Δ	With Na^+	Without Na^+	Δ
Primary fractions 2:	33·55 ± 3·53	21·28 ± 2·28	12·27	31·30 ± 2·20	19·60 ± 3·06	11·70
A	11·94 ± 1·28	11·94 ± 1·28	—	11·53 ± 1·65	11·53 ± 1·65	—
B	11·88 ± 2·52	12·00 ± 2·73	—	12·59 ± 2·77	11·89 ± 1·70	—
C	23·74 ± 3·84	11·42 ± 2·23	12·32	18·19 ± 3·57	10·29 ± 3·91	7·90
D	30·30 ± 4·53	21·75 ± 3·65	8·55	30·58 ± 6·65	19·34 ± 3·26	11·24
E	41·82 ± 4·39	24·47 ± 3·61	17·35	41·20 ± 6·24	24·69 ± 3·16	16·51
F	38·70 ± 4·18	26·86 ± 5·10	11·82	40·39 ± 4·13	28·52 ± 2·63	11·87

Values are the results of six experiments (mean ± S.D.).

A marked decrease of Na^+K^+ATPase specific activity (μmoles/mg/protein/hr) was observed in fraction C, smooth membranes (Table 4), but no change was found in the other fractions.

Hazon and Toschi[22] found acetylcholinesterase activity associated with microsomal membranes rather than with ribosomes. The results of Table 5 confirm this; treatment with the drug resulted in slight decrease of AChE activity in fraction C, smooth microsomal membranes, and a more pronounced decrease in fraction E which contains the membrane fraction originating from the synaptic regions.

TABLE 5. ACETYLCHOLINESTERASE (AChE) ACTIVITY IN THE FRACTIONS PREPARED BY DENSITY GRADIENT CENTRIFUGATION

Fraction	AChE (μmoles acetylthiocholine converted/mg of protein/hr)	
	Control	Treated
Primary fractions 2	3·500 ± 0·605	3·316 ± 0·684
A	4·798 ± 0·634	4·882 ± 0·592
B	2·967 ± 0·703	3·109 ± 0·703
C	3·269 ± 0·168	2·862 ± 0·265
D	5·296 ± 0·500	5·189 ± 0·772
E	5·380 ± 0·304	4·053 ± 0·261
F	1·857 ± 0·131	1·822 ± 0·340

Values are the results of six experiments (mean ± S.D.).

DISCUSSION

Chronic administration of a drug which acts as a tranquilizer at dose of 5 mg/kg and as a convulsant at higher doses, 10–20 mg/kg, did not appear to affect the protein content of the brain significantly. Only slight changes in the content of RNA were observed in the ribosome fraction and only a small increase in the protein of membrane fractions occurred. It should be noted that the estimations were of total protein and total RNA; it is possible that changes may have occurred in individual macromolecular species.

Numerous reports have described changes in protein synthesis and in RNA metabolism resulting from specific stimuli (administration of phenothiazines or metrazol) or in behavioural experiments. Shuster and Hannan[23] reported the inhibition of protein synthesis by chlorpromazine and Raghupathy *et al.*[24] have shown that phenothiazines *in vitro* decrease protein synthesis in brain and that this inhibition results from an interaction between the drug and the soluble transferases.

Talwar *et al.*[25] reported a decrease in the RNA content of rat brain during the tonic phase of metrazol convulsions but Chitre *et al.*[26] suggested that the brain after convulsion would be synthesising RNA at an accelerated pace to regain the normal levels.

It is difficult to relate the relevance of altered brain function resulting from administration of a tranquillizer to the changes and enzymic activity observed in this study. Whereas slight increase in the total protein of membrane fractions occurred, the activities of enzymes normally associated with these fractions were slightly decreased.

In order to clarify this, a study of the changes which may occur during the 3-week period of drug administration, and of the effects on turnover rates of RNA and protein, seems indicated.

This study must also include an investigation of possible accumulation of the drug in rat brain after repeated dosage in similar fashion to some phenothiazines as related by Mahju and Maićkel,[27] and the possibility of binding of the drug to specific molecules in the brain.

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