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SHORT COMMUNICATIONS

A preliminary biochemical examination of micrencephalic rat brains*

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THE ADMINISTRATION of methylazoxymethanol[1,2] or its acetate[3,4] to pregnant rats results in offspring that are micrencephalic. The action of this teratogen as a methylating agent[5] of nucleic acids and protein might be expected to yield surviving brain cells with modified biochemical properties in addition to exerting a lethal effect that results in the reduction in the total number of brain cells.[6] For this reason, we have started to study the biochemical status of these micrencephalic brains.

Long–Evans rats were bred, reared and injected as previously described,[4] except that the day of injection of methylazoxymethyl acetate into the experimental mothers was restricted to the fifteenth day of gestation. Offspring from both groups were sacrificed at 35 days of age by decapitation and the brains immediately removed and separated into two mirror-image parts by a cut through the median sagittal plane. Each piece was weighed, quick-frozen on dry ice and stored at −70° until analyzed.

The right half of each brain was processed as described by Penn and Suwalski[7] for the determination of DNA, RNA and protein. DNA was measured by the modified diphenylamine reaction.[8] The RNA which was separated from the DNA during a hydrolysis step in the isolation scheme was determined by the modified orcinol reaction of Lin and Schjeide.[9] Protein was measured on the residue remaining after ammonium hydroxide solubilization of the DNA by employing the method of Lowry *et al.*[10] and using bovine serum albumin as a standard.

TABLE 1. DNA, RNA AND PROTEIN CONTENT OF CONTROL AND MICRENCEPHALIC BRAINS*

	Control		Experimental		Probability
Sex	Male	Female	Male	Female	
No. of animals	7	9	7	7	
Brain wt (g)	1·704 ± 0·037		1·105 ± 0·061		<0·001
		1·625 ± 0·028		1·027 ± 0·077	<0·001
Total DNA (mg)	2·010 ± 0·058		1·613 ± 0·086		<0·001
		1·946 ± 0·052		1·565 ± 0·313	<0·005
DNA per unit fresh wt (mg/g)	1·179 ± 0·023		1·471 ± 0·071		<0·001
		1·198 ± 0·023		1·512 ± 0·255	<0·01
Total RNA (mg)	5·538 ± 0·266		2·765 ± 0·314		<0·001
		5·032 ± 0·463		2·601 ± 0·099	<0·001
RNA per unit fresh wt (mg/g)	3·258 ± 0·155		2·518 ± 0·379		<0·001
		3·095 ± 0·149		2·540 ± 0·160	<0·001
Total protein (mg)	237 ± 13		144 ± 25		<0·001
		194 ± 12		116 ± 15	<0·001
Protein per unit fresh wt (mg/g)	136 ± 7·7		129 ± 14·9		<0·5
		119 ± 5·4		114 ± 7·2	<0·05

* Values represent means ±S.D.

It is evident from the results in Table 1 that the micrencephalic brains contained approximately 20 per cent less total DNA than the control brains, thereby confirming the histological observation of a reduced number of cells in the experimental brains. This decrease compares favorably with the 25 per cent reduction in DNA recently reported by Matsumoto *et al.*[11] Since all parts of the brain have

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been shown to be reduced in size but to varying degrees, the entire half of the brain was analyzed rather than the cerebral hemisphere alone. While no physical measurements have been made on the cerebellums from the micrencephalic brains, it was apparent that this part of the brain which contains polyploidal cells[12] was not reduced to the same extent as the cerebrum. This condition probably accounts for the greater DNA content per gram of wet tissue in the experimental brains.

RNA, on the other hand, was significantly reduced in total and per unit weight of brain; possibly due to the chemical modification of DNA and its resultant effect on RNA synthesis. Nagata and Matsumoto[13] have shown, in a short-term experiment, that the administration of tritium-labeled methylazoxymethyl acetate to pregnant rats yielded isolatable tritium containing methylated DNA and RNA from the brains of the fetuses. Methylation had occurred preferentially at the 7-position of guanine and it has been shown that comparable methylation of calf thymus DNA resulted in its loss of template activity for DNA and RNA polymerase.[14]

Total protein was reduced in the micrencephalic brains, but the quantity per unit of wet tissue was equivalent to control protein. Considering the chemical processes employed in the isolation procedure, this protein may be of structural origin and may not reflect the total *in vivo* cellular protein.

Pursuant to examining enzyme levels in control and experimental animals, 24-hr urines from both groups were screened for any obvious metabolic irregularity. Paper chromatographic procedures customarily employed in the clinical screening of 24-hr specimens failed to detect any compositional differences in amino acids or carbohydrates.

A comparison of the general physical appearance of the micrencephalic brains produced by methylazoxymethyl acetate with photographs of micrencephalic brains induced by irradiation[15] revealed striking similarities and led to the speculation that biochemical similarities might also exist between them. While the mode of action is not the same, both methods involve the modification of nucleic acids.

It has been reported that gamma irradiation on the thirteenth day of gestation resulted in micrencephalic offspring with reduced cerebral lactate dehydrogenase (L-lactate: NAD reductase, EC 1.1.1.27) activity.[16] The isoenzyme pattern was also altered and was characterized by an increase in the percentage of isoenzymes 1 and 2, and a reduction in 4 and 5. Based on these observations, a 5% homogenate of the left half of each brain was prepared in 0·25 M sucrose at 0° for subsequent enzymatic assays. A portion of the homogenate was diluted 20-fold with a 0·1 M phosphate buffer, pH 7·4, for the measurement of lactate dehydrogenase (LDH) activity by the method of Kornberg.[17] The NAD formed was measured fluorometrically[18] using an Aminco–Bowman spectrofluorometer. The results in Table 2 were expressed as the number of micromoles of NAD formed per minute per gram fresh weight.

The LDH isoenzymes were separated by cellulose acetate electrophoresis[19] of an 8-μl sample of the original homogenate. It was found that a 30-min running time at 200 V was the most satisfactory condition for good separation and reproducibility. The isogram was analyzed by eluting the bands in 1 ml acetone and measuring the absorption at 400 nm with a Beckman DU spectrophotometer.

From the results in Tables 2 and 3, it is apparent that not only was the activity of LDH reduced but the isoenzyme pattern exhibited an increase in isoenzyme 1 and a reduction in 5. This change in isoenzyme distribution is a normal phenomenon that accompanies an increase in animal age.[20] The magnitude of the change, however, is indicative of a much older animal. This accelerated maturation of the LDH isoenzymic pattern in the developing brain has also been observed in animals subjected to postnatal X-irradiation.[21] The reduction of total LDH activity observed in this experiment may be a further manifestation of premature maturation, for Kanungo and Singh[22] have shown that LDH activity reaches a maximum in rat brain at 30 weeks and then slowly declines. An examination of micrencephalic brain LDH activity during development would be necessary to validate this conjecture.

To assess the effect of the teratogen on acid hydrolases, we chose to measure β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) activity in this initial experiment rather than the conventional lysosomal marker, acid phosphatase. This choice was based on the fact that we are routinely assaying for this enzyme in our laboratory, and it is an enzyme that is prevalently associated with several mental retardation conditions. β-Galactosidase was measured on 0·5 ml of the homogenate diluted with an equal volume of 200 mM acetate buffer, pH 5·0, containing 2·5 μmoles *o*-nitrophenyl-β-D-galactopyranoside.[23] The reaction was terminated by the addition of glycine-carbonate buffer, pH 10·7, and the color intensity was read with a Beckman DU at 420 nm. Specific activity was recorded as the number of millimicromoles of substrate hydrolyzed per hour at 37° per milligram of protein. Surprisingly the specific activity for β-galactosidase (Table 2) in the experimental brains exceeded the activity in control brains. Drawing comparisons again with irradiation, it is known that the latter condition enhances the activity of lysosomal enzymes, apparently by affecting the organelle's membrane.[24] Similarly, dosing with dimethylnitrosamine, another methylating agent, produced increased levels of plasma β-glucuronidase and acid phosphatase.[25] Both these results and those due to irradiation were obtained in short-term experiments, lasting less than 24 hr, thereby making mechanistic type comparisons impossible.

TABLE 2. SPECIFIC ACTIVITIES OF LACTATE DEHYDROGENASE, β-GALACTOSIDASE AND GLUTAMATE DECARBOXYLASE IN CONTROL AND MICRENCEPHALIC RAT BRAINS*

	No. of animals		Lactate dehydrogenase (μmoles NAD/min/g fresh wt)		β-Galactosidase (nmoles/hr/mg protein)		Glutamate decarboxylase (μmoles/hr/g fresh wt)	
Group	Male	Female	Male	Female	Male	Female	Male	Female
Control	7	9	46·8 ± 8·2	43·2 ± 3·7	51·8 ± 7·4	51·1 ± 7·1	9·39 ± 2·75	10·21 ± 3·92
Experimental	7	7	35·9 ± 4·0	39·1 ± 4·0	58·5 ± 5·1	59·1 ± 4·0	5·80 ± 1·39	5·59 ± 1·90
Probability			<0·001	<0·1	<0·1	<0·02	<0·01	<0·01

* Values represent means ±S.D.

TABLE 3. LACTATE DEHYDROGENASE ISOENZYME COMPOSITION IN CONTROL AND MICRENCEPHALIC RAT BRAINS*

Group	No. of animals		Isoenzymes (% of total activity)				
	Male	Female	1	2	3	4	5
Control	5	9	19·4 ± 2·2	18·7 ± 2·9	21·1 ± 3·0	29·2 ± 3·1	13·1 ± 1·5
Experimental	4	4	32·6 ± 9·5	16·2 ± 3·9	16·2 ± 3·9	25·9 ± 5·2	9·8 ± 2·7
Probability			<0·001	<0·2	<0·01	<0·1	<0·001

* Values represent means ±S.D.

All animals sacrificed in our laboratory by perfusion are anesthetized with sodium pentobarbital. The initial reaction of micrencephalic animals to the drug is a violent convulsive type careening about the cage before sedation. Tapia *et al.*[26] have suggested that reduced levels of glutamate decarboxylase (L-glutamate 1-carboxylyase, EC 4.1.1.15) are associated with various types of convulsions, and this led us to assay for this enzyme by the fluorometric method of Lowe *et al.*[27] From the results in Table 2, it is apparent that glutamate decarboxylase activity is reduced significantly. How this condition relates to the forementioned behavior and to the apparent hyperactivity of the experimental animals are questions yet to be answered.

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Effect of cholinesterase inhibitors on active potassium influx in monkey erythrocytes

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The role of the acetylcholinesterase present in erythrocytes is still unknown. However, a number of reports in the literature have studied the possible involvement of acetylcholinesterase in ion transport and ion permeability in these cells.[1–4] In all these studies, uptake or release of sodium or potassium from erythrocytes were measured after prolonged incubation, i.e. for several hr or longer. In the following we report the effect of various cholinesterase inhibitors on active K^+-influx in monkey erythrocytes. The technique utilized permitted influx determination at short times (5–30 min) after exposure of the erythrocytes to the reagent tested.[5]

The compounds examined included three inhibitors of cholinesterase which inhibit the enzyme by covalently blocking the serine at the active site, diisopropylfluorophosphate, eserine and neostigmine, the substrate acetylcholine and two quaternary ammonium compounds, decamethonium and butyrylcholine, which can bind to the enzyme reversibly.

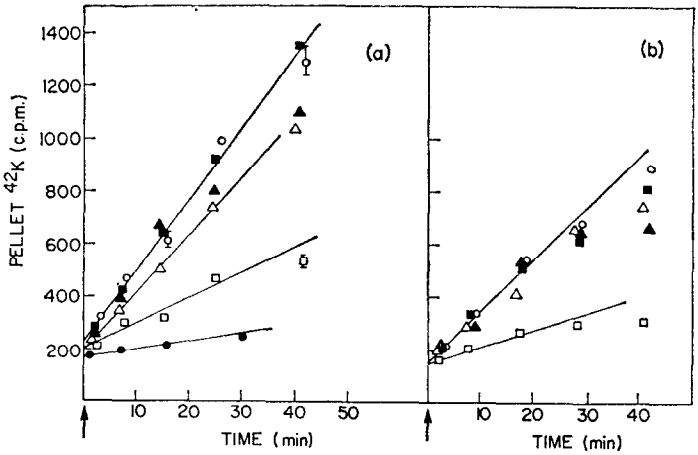

Fig. 1. Kinetics of uptake of ^{42}K by treated and control monkey erythrocytes. Samples were removed and the erythrocyte pellet separated and counted as described in the text. (a) ○, control; □, 0·1 mM eserine; △, 1 mM diisopropylfluorophosphate; ■, 0·075 mM neostigmine; ▲, 1 mM decamethonium; ●, 1 mM ouabain. (b) ○, control; □, 1·2 mM eserine; △, 1·2 mM diisopropylfluorophosphate; ■, 1·2 mM butyrylcholine; ▲, 27 mM acetylcholine. ↑, at zero time ^{42}K was added to the suspension.

In the experiments described below, Rhesus monkey erythrocytes from freshly drawn blood were washed twice and resuspended (about 9% haematocrit) in 4 mM KCl–136 mM sodium phosphate pH 7·3. Aliquots of 0·9 ml were then incubated in 13 mm diameter tubes at 37° with gentle shaking. After 2 min, 50 μl of a solution containing the appropriate reagent was added to each tube. Eight min later, 0·1 ml aliquots were withdrawn for cholinesterase activity determinations by the method of Ellman *et al.*[6] A 50 μl aliquot of a ^{42}K solution was then added to each tube. This solution contained 10 mM KCl–140 mM NaCl, pH 7·3 and contained about 400 μCi/ml.

The uptake of the radioisotope by the cells was obtained from radioactivity measurements of