5. Doherty, D. G., *in* "Radiation Protection and Recovery," (A. Hollaender, ed.), p. 45. MacMillan (Pergamon), New York, (1960).

6. McIsaac, W. M., Parke, D. V., and Williams, R. T., Biochem. J. 70, 688 (1958).

7. Jandl, J. H., Files, N. M., Barnett, S. B., and MacDonald, R. A., J. Exptl. Med. 122, 299 (1965).

8. Lajtha, L. G., Gilbert, C. W., Porteous, D. D., and Alexanian, R., Ann. N. Y. Acad. Sci. 113, 742 (1964).

9. Till, J. E. and McCulloch, E. A., Radiation Res. 14, 213 (1961).

10. Hodgson, G. S., Acta Physiol. Latinoam. 12, 365 (1962).

11. Smith, L. H. and Vos, O., Intern. J. Radiation Biol. 5, 461 (1962).

Received July 1, 1968. P.S.E.B.M., 1968, Vol. 129.

Transplacental Chemical Induction of Microencephaly in Two Strains of Rats. I. (33404)

MARIA SPATZ AND GERT L. LAQUEUR

Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Maryland 20014

In July 1967, 4 litters of Fischer rats, 14 months old, were sacrificed and unexpectedly found to have various degrees of microencephaly. The assumption was that all mates had been equally and simultaneously exposed to an etiologic agent because the reduction in weight of the brain was strikingly uniform within each litter.

The four litters were parts of a larger study in which the attempt was made to correlate site and frequency of transplacentally induced neoplasms with the stage of fetal development at the time of exposure to methylazoxymethanol (MAM), the aglycone of cycasin and a potent carcinogen. Evidence for transplacental passage of both the glucoside cycasin and its aglycone provided the necessary information to assume a direct chemical interaction between these compounds and embryonic or fetal tissue (1).

The protocols indicated that the respective mother rats had received a dose of MAM equivalent to 20 mg/kg of body weight which had been administered intraperitoneally in three equally divided doses on days 14, 15, and 16 of gestation.

Experiments were started to examine reproducibility, strain dependency, optimal dose and time of fetal development for successful induction, postnatal growth of the brain, effects of mating of microencephalic sisters and brothers on the brain of their progeny, and possible intellectual deficits in microencephalic rats in conjunction with anatomical alterations. It is the purpose of this paper to describe the experimental conditions under which microencephalic litters were produced with regularity in two strains of rats, to report on the growth of the brain during postnatal life and on the result of sister–brother matings of microencephalic rats.

Materials and Methods. Animals. Two strains of rats, the Fischer and the Osborne-Mendel strains, were used. Young males and females were obtained at weanling age from the animal production facilities at the National Institutes of Health. After they had reached maturity, they were bred in our laboratory, and the day on which spermatozoa were identified in the vaginal smear was designated as day 1 of pregnancy. The impregnated females were separated and housed in individual plastic cages. The rats were weighed on day 14 of pregnancy unless stated otherwise and the total dose of MAM was calculated on the basis of 20 mg/kg of body weight. This amount was administered intraperitoneally either in 3 equally divided doses on days 14, 15, and 16 of pregnancy or, in later experiments, as the entire amount on either day 13, 14, 15, or 16 of pregnancy to

TABLE I. Effects of MAM, Given Intraperitoneally to Pregnant Fischer Rats in Divided Doses on Day 14, 15, and 16 of Gestation, on the Weight of the Brain in the Offspring.

	Maternal dose of MAM (mg/kg of body wt.)	Litter identification	No. of rats surviving 14 months	Mean wt. of brain (g ± SE)	Significance of difference between mean brain wt. of experimentals and age controls
Controls	—	RA_2, RF_2	15	1.995 ± .025	
Experimentals	20	RA 28 and 30	7	1.650 ± .028	<.001
	20	RA 31	9	1.334 ± .029	<.001
	20	RA 32	5	1.778 ± .052	<.001

determine the optimal time for successful induction of microencephaly. All rats were fed commercial Purina laboratory chow and had access to tap water without restrictions. The day of littering was noted and the young were counted.

Control rats of the two strains were handled in an identical manner except that they remained either uninjected or received an equivalent amount of saline in which MAM had been administered to the experimentals. Since intraperitoneal injections of saline did not affect pregnancy or litter size and did not produce cerebral abnormalities in the offsprings, the controls are treated as one group.

The young were used for various purposes as indicated in Tables II and III. At autopsy, the bony calvarium was removed and the entire head with the brain *in situ* was fixed in 10% neutral formalin for 24 hr. The brain was then removed and placed in fresh fixative for an additional 24 hr before weighing. Fresh weights were also taken of the liver and kidneys at the time of autopsy to determine whether the induced microencephaly was a part of a generalized microsplanchnia or whether it had developed as an independent lesion.

Chemicals. Methylazoxymethanol (MAM) was prepared from cycasin in our laboratory using the method of Kobayashi and Matsumoto (2). The MAM acetate, a synthetic compound, was given to us by Dr. Matsumoto who had performed the original synthesis (3).

Results. Data obtained in the original experiment is presented in Table I and an example of the gross appearance of the brain of a microencephalic rat together with its age control is shown in Fig. 1 in which the experimental animal is one of the nine long-term survivors of litter RA 31. Table I shows considerable variation in the brain weights between the litters, but statistical treatment of the data demonstrates that the mean brain weight is uniformly smaller than that of age controls, p being less than .001 in every instance.

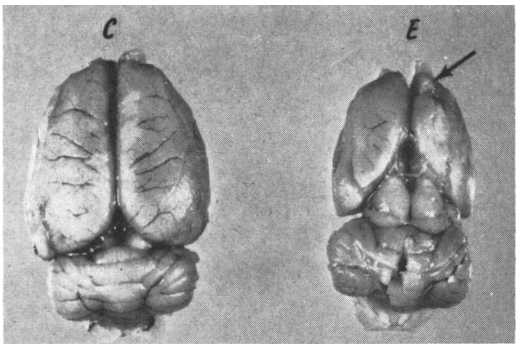


FIG. 1. Dorsal view of brains of microencephalic rat (E) and of control (C). Arrow indicates site of glioma in forebrain.

The reduction in brain weight essentially was due to a diminution of both cerebral hemispheres which resulted in broad exposure of the corpora quadrigemina. There was no obvious increase in cerebrospinal fluid in the 14 month-old rats and cerebellum, pons, and medulla did not appear affected by the condition. There was, however, an associated narrowing in width of the parietal bone of 1–2 mm on each side. Gliomas were found in four rats of the original experiment.

Reproducibility and strain dependency were tested in 12 pregnant Fischer and 3

TABLE II. Significance of the Difference Between the Means of the Weight of the Brain in Rats Exposed *in Utero* to Methylazoxymethanol and Their Respective Controls.

Strain of rats	Post-partum (days)	Controls			Experimentals			Differences between means	
		No. of rats	Mean brain wt. (g)	(SD)	No. of rats	Mean brain wt. (g)	(SD)	t	p
Fischer	1	4	0.287	±.029	5	0.214	±.012	5.447	<.001
	4	7	0.553	±.065	6	0.405	±.079	3.700	<.01
	7	7	0.846	±.110	6	0.660	±.085	3.249	<.01
	14	13	1.447	±.072	8	1.252	±.110	11.079	<.001
	28	12	1.675	±.083	31	1.278	±.091	13.233	<.001
	60	6	1.772	±.067	7	1.264	±.108	12.650	<.001
	135	12	1.843	±.073	24	1.306	±.090	17.900	<.001
	400	15	1.955	±.099	9	1.334	±.086	15.525	<.001
Osborne-Mendel	28	62	1.818	±.080	25	1.242	±.142	24.303	<.001

pregnant Osborne-Mendel rats which received a total dose of 20 mg/kg of body weight in 3 divided doses on day 14, 15, and 16 of gestation. These pregnancies resulted in 89 newborn Fischer and 25 newborn Osborne-Mendel rats all of which proved to be microencephalic at autopsy. Twenty-six of the 89 Fischer rats were reserved for intellectual evaluation together with saline injected controls when 2 months old. The remaining rats and others treated identically were used to study the growth of the postnatal brain as shown in Table II and Fig. 2. Included in Table II are the results of the statistical treatment of the data indicating that the brain weights of the experimentals were significantly below those of the controls at all postpartum intervals. It should be emphasized that, in obtaining these data, care was taken to have representatives of as many litters as was possible for each day of weighing. Table II shows furthermore an equally striking microencephaly in Osborne-Mendel rats when treated in an identical manner. Obviously, induction of microencephaly was not only reproducible but developed also in a different noninbred strain of rats. In plotting graphically the absolute weights of the brains at various times after birth (see Fig. 2), it became apparent that the average increase in brain weight was similar in the experimentals (1.048 g) and controls (1.160 g) for the first 14 postpartum days. Beginning with day 14, there was little further weight increase among the microencephalic rats whereas the brain continued to grow in the controls though at a much reduced rate.

While these studies were underway, preliminary observations suggested that microencephaly might be inducible with a single intraperitoneal injection of MAM and this possibility was explored. Table III, which summarizes the results, shows that day 15 of pregnancy was apparently the optimal time for the induction of microencephaly with a single dose of MAM. The mean brain weight

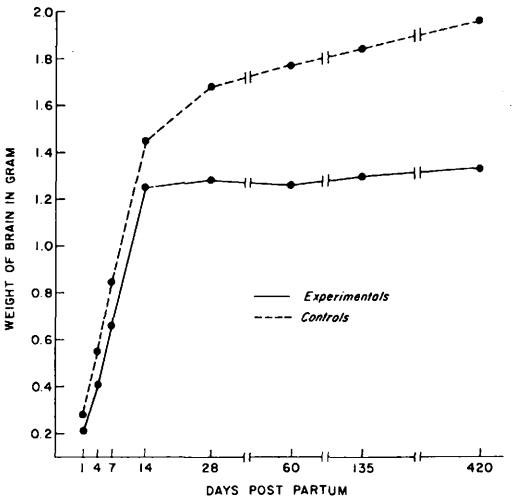


FIG. 2. Postnatal growth of brain of MAM-exposed Fischer rats and of their respective age controls.

TABLE III. Comparison of the Effect of MAM, Given Intraperitoneally Either in Divided or as Single Doses to Fischer Rats at Various Days of Pregnancy, on the Weight of the Brain of Their Respective Young on Day 28 of Life (total dose: 20 mg/kg of body wt.).

Groups of rats	No. of rats/group	Fetal development (days)	No. of maternal injections	Mean wt. of brain (g ± SE)	Value of p or mean wt. significantly different from: Controls	3-Day experimentals
Controls	12	—	—	1.675 ± .024		
Experimentals	31	14, 15, 16	3	1.278 ± .016	S[a]	
	15	13	1	1.390 ± .016	S	S
	24	14	1	1.285 ± .023	S	NS[b]
	17	15	1	1.168 ± .022	S	S
	8	16	1	1.290 ± .032	S	NS
	6	16	1	1.633 ± .043	NS	S

[a] S (significant): $p = <.05$.
[b] NS (not significant): $p = .05$ or larger.

was significantly smaller than that of the group which had received the same amount in divided doses during 3 successive days ($p = <.001$). This difference was not significant, however, for the rats injected on day 14 of fetal life and significant in the opposite direction for the rats injected on day 13 of fetal growth in that their brains were larger than those obtained when the divided treatment schedule was used. The weight of the brains from rats exposed *in utero* on day 16 of pregnancy fell into two distinct groups and both are listed in Table III. The difference between the two mean weights is striking and may well reflect the difficulty in correctly knowing the time of impregnation of the female rats and thus the onset of pregnancy. Further experiments will be done to clarify this particular point. Recent studies in which equivalent amounts of MAM were administered intravenously have shown that this route was equally effective in producing microencephaly. There was no effect of MAM on the weights of the liver and kidneys in the microencephalic rats, thus excluding an associated microsplanchnic condition.

Fertility of microencephalic rats was not significantly impaired when sister–brother matings were attempted. Twenty-seven young were obtained from four such matings. The young were sacrificed when 28 days old as were the respective parents and grandparents. There were no obvious malformations in the offspring of microencephalic parents and the brain weights were not significantly different from those of 28-day control rats, p being larger than .05 (Fig. 3).

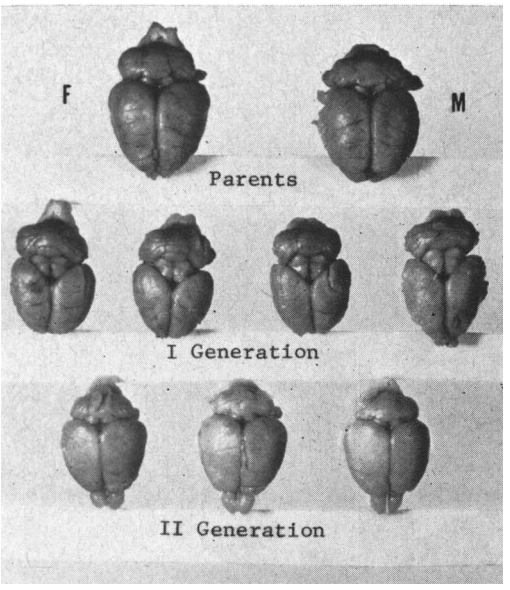
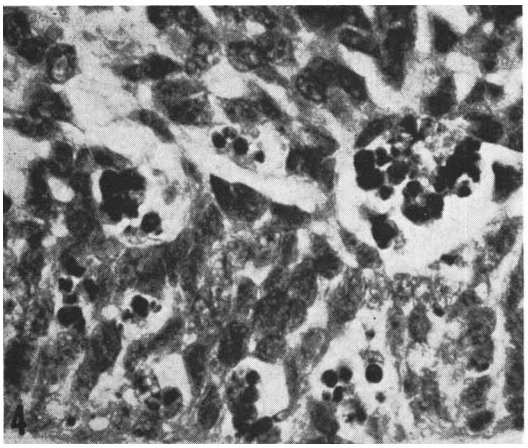


FIG. 3. Brains from 3 generations of rats: (upper row) parental generation in which the female (F) received MAM on days 14, 15, and 16 of pregnancy; (middle row) offspring showing uniformly microencephaly; (bottom row) offspring with normal brains resulting from sister–brother mating of microencephalic rats shown in middle row.



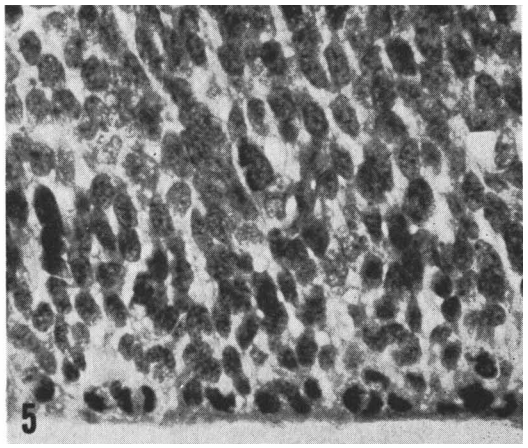


FIG. 4. Reduction in width of subependymal neuroblast with foci of necrosis in 17-day old fetus, 48-hr after a single dose of MAM. Hematoxylin and eosin stain; × 500.

FIG. 5. Same area from a 17-day old control fetus. Hematoxylin and eosin stain; ×500.

Preliminary morphologic studies indicate that MAM produced extensive necrosis of cells derived from neuroblasts lining the ventricular spaces and involving cells between the cavities and the outer cortical zone (Figs. 4 and 5). Similar though less severe necrosis was noted among the retinal cells in serial sections of a 17-day old fetus which had been exposed on day 15 to a single dose of MAM. Choroid plexus and caudal areas including spinal cord were free of necrotic lesions. Details of the pathologic changes in these fetuses will be reported separately after completion of our studies.

Discussion. The experiments present evidence for a transplacental, chemical induction of microencephaly, a condition which was consistent with long life and unaccompanied by other malformations. The chemical, methylazoxymethanol,

$$\begin{array}{c} O \\ \uparrow \\ CH_3 - N = N - CH_2OH \end{array}$$

a compound of low molecular weight, is readily soluble in aqueous solutions, highly unstable and reactive by methylating bases of RNA and DNA both *in vitro* (4) and *in vivo* (5). The same compound was previously shown to be a mutagen (6,7) and teratogen (8) and its general carcinogenic properties have already been well established (9,10).

The microencephaly was inducible with a single intraperitoneal or intravenous administration of MAM at the beginning of the third trimester of pregnancy. Without altering litter size or survival of the young, a dose of 20 mg/kg of body weight consistently produced microencephaly.

A striking feature of the chemically induced microencephaly was its uniformity among littermates. Differences in the degree of microencephaly between litters were most likely the result of variation in timing the injection which was based on considering the day on which spermatozoa were found in the vaginal smear, as day 1 of pregnancy. The smears were taken at about 9 a.m. and thus the animals were unobserved for the preceding 15–16 hr with fertilization possible at any time during that period. Thus, the difference in brain weights between litters which was noted on day 16 and illustrated in Table III can be explained, particularly since microencephaly most readily occurred on day 15, but not when MAM was given on day 17 of fetal development.

Although our studies on the biochemical and pathologic alterations during the development of microencephaly with MAM are still in progress, data available in the literature on radiation induced cerebral malformations (11, 12) indicate that microencephaly induced by X-ray irradiation could be obtained from days 13 to 18 whereas the MAM induced microencephaly was limited to days

13, 14, and 15 and perhaps early on day 16 of fetal development. It would seem possible that this difference in days during which the fetal brain was affected in a similar way by the two procedures was due to the fact that X-ray irradiation produced its effect very rapidly and directly whereas the administered chemical needed to be absorbed and carried to the developing brain by the fetal circulation. It is of interest, however, that the principal lesion appears to be similar in the two conditions in that extensive necrosis of cells derived from the neuroblast lining the lateral ventricles is found. Scattered necrotic cells were also observed in retinal cells but not in the spinal cord. A study of the details of the sequential changes in the developing brain under the influence of MAM is well under way and will be reported separately, as will be the evaluation of the intellectual status of the microencephalic rats by our collaborators, Drs. Haddad and Rabe of the New Jersey Bureau of Research in Neurology and Psychiatry in Princeton.

The relationship of the necrotizing process in the brain to the later development of gliomas as observed in four microencephalic rats is at present uncertain as is the development of late cancer in the liver of rats which in the course of acute cycasin or MAM intoxication developed extensive centrilobular liver-cell necrosis.

The cause for the sharp break in the growth of the brain in microencephalic rats at day 14 and thereafter is presently being investigated. It is of interest that the break occurs at a time when the cerebral lipid content significantly increases in normal animals (13, 14) in association with myelination of the fiber systems (15). The extensive necrosis noted among the cells destined to participate in the formation of the cortex suggests a marked reduction in their numbers following MAM administration. Whether MAM does affect, in other (biochemical) ways, myelin formation remains to be determined.

Summary. An experimental model for successful chemical induction of microencephaly is described. Methylazoxymethanol, the aglycone of cycasin, when administered to rats at a dose of 20 mg/kg of body weight on day 14 or 15 of pregnancy, uniformly produces microencephaly in all litter mates. This effect occurs independent of the strain of rats used and is highly reproducible. The condition is not associated with microsplanchnia and appears to be limited to the cortical hemispheres of the brain. Preliminary pathologic studies indicate a marked cytotoxic effect of the chemical on the neuroblast. Gliomas have occurred in about 10% of microencephalic rats permitted to live beyond 1 year.

1. Spatz, M. and Laqueur, G. L., Proc. Soc. Exptl. Biol. Med. **127**, 281 (1968).

2. Kobayashi, A. and Matsumoto, H., Arch. Biochem. Biophys. **110**, 373 (1965).

3. Matsumoto, H., Nagahama, T., and Larson, H. O., Biochem. J. **95**, 13c (1965).

4. Matsumoto, H. and Higa, H. H., Biochem. J. **98**, 20c (1966).

5. Shank, R. C. and Magee, P. N., Biochem. J. **105**, 521 (1967).

6. Smith, D. W. E., Science **152**, 1273 (1966).

7. Teas, H. J. and Dyson, J. G., Proc. Soc. Exptl. Biol. Med. **125**, 988 (1967).

8. Spatz, M., Dougherty, W. J., and Smith, D. W. E., Proc. Soc. Exptl. Biol. Med. **124**, 476 (1967).

9. Laqueur, G. L. and Matsumoto, H., J. Natl. Cancer Inst. **37**, 217 (1966).

10. Laqueur, G. L., McDaniel, E. G., and Matsumoto, H., J. Natl. Cancer Inst. **39**, 355 (1967).

11. Hicks, S. P., D'Amato, C. J., and Lowe, M. J., J. Comp. Neurol. **113**, 435 (1959).

12. Hicks, S. P., Physiol. Rev. **38**, 337 (1958).

13. Koch, W. and Koch, M. L., J. Biol. Chem. **15**, 423 (1913).

14. Folch, J., Casals, J., Pope, A., Meath, J. A., LeBaron, F. N., and Lees, M., Prog. Neurobiol. **4**, 122 (1959).

15. Watson, J. B., Dissertation, University of Chicago (1903).

Received July 8, 1968. P.S.E.B.M., 1968, Vol. 129.