Br. J. Pharmac. (1975), 54, 33-39

A COMPARISON OF THE EFFECTS OF CYTOSINE ARABINOSIDE AND ADENINE ARABINOSIDE ON SOME ASPECTS OF BRAIN GROWTH AND DEVELOPMENT IN THE RAT

B.P.F. ADLARD, J. DOBBING & JEAN SANDS

Department of Child Health, University of Manchester, The Medical School, Stopford Building, Oxford Road, Manchester M13 9PT

1 Treatment of pregnant rats with cytosine arabinoside (ara-C, 50 mg/kg, i.p.) at 14 days of gestation severely impaired both prenatal and postnatal whole brain growth in their offspring, although the cerebellum was relatively less affected than whole brain.

2 Rats treated at 5 days of age with ara-C (250 mg/kg, i.p.) showed an impairment in growth of the cerebellum relative to the rest of the brain.

3 Adenine arabinoside (ara-A) treatment, either prenatally or postnatally, had negligible effect on brain growth, even at doses considerably higher than those of ara-C.

4 Adult rats, previously treated with ara-C (50 mg/kg, i.p.) at 14 days of gestation, showed an impairment in discrimination learning when tested in a water T-maze.

5 These results are discussed in relation to the proposed use of ara-C or ara-A as antiviral agents, particularly against intrauterine infection with cytomegalovirus.

Introduction

Congenital infection with cytomegalovirus may have serious consequences for brain development (Hanshaw, 1966; McCracken, Shinefield, Cobb, Rausen, Dische & Eichenwald, 1969; Berenberg & Nankervis, 1970), and there are increasing suspicions that, in some cases, microcephaly and mental retardation may result even when the immediate clinical symptoms are negligible (Hanshaw, 1966; Stern, Elek, Booth & Fleck, 1969). Many infants with congenital infection have probably been exposed to the virus during a large part of intrauterine development. There is therefore a quest for suitable antiviral agents to treat the condition during the neonatal period, and perhaps also, during pregnancy.

Cytomegalovirus is a DNA virus of the herpes group whose replication may be prevented by inhibitors of DNA synthesis (Sidwell, Arnett & Brockham, 1970) and in particular by cytosine arabinoside (ara-C). Ara-C is an antimetabolite which acts primarily as an inhibitor of DNA polymerase and has been used, with varying success, to treat cytomegalovirus infection in the neonatal period (Kraybill, Sever, Avery & Movassaghi, 1972; McCracken & Luby, 1972). The toxicity of ara-C is well known from its use in leukaemia, and in a developing organism this drug might in particular affect neuronal multiplication.

It is therefore necessary to assess whether ara-C, in the doses employed therapeutically, has any adverse effect on brain development. Such effects might be most marked if the drug were given during the major period of neurogenesis, 10 to 18 weeks of gestation in man (Dobbing & Sands, 1973) or 14 to 21 days of gestation in the rat (Croskerry, Smith, Shepard & Freeman, 1973).

We have investigated the effects of ara-C, given at either 14 days of gestation or 5 days of postnatal age, on some aspects of brain growth and development in the rat. At 5 days of age the rat brain is in a phase of development comparable with the human brain at term (Dobbing & Sands, 1973). The corresponding effects of adenine arabinoside (ara-A) have also been studied. This substance, probably of lower toxicity than ara-C (Kurtz, Fisken, Kaump & Schardein, 1969), has been suggested (Miller, Dixon, Ehrlich, Sloan & McLean, 1969; Schabel, 1970; Plotkin, 1972) as an alternative antiviral agent.

Methods

Animals

Rats of the Lister black and white hooded strain

were fed a good quality diet (Breeding Diet for Rats and Mice, Oxoid Ltd.). Maternal animals had previously reared either one or two litters. The day of mating (day 0) was determined by examination of vaginal smears for sperm.

Drugs

Cytosine arabinoside (1-β-D-arabinofuranosylcytosine; Upjohn Ltd.) was administered as a solution in 0.9% w/v NaCl solution (saline). Adenine arabinoside (9-β-D-arabinofuranosyladenine; Parke, Davis and Co.), which is sparingly soluble in water, was given as a suspension in saline. Control animals received saline only. Drugs and saline were injected intraperitoneally at a dose of 10 ml/kg in both pregnant and postnatal rats, and were always administered at approximately 10 h 00 minutes.

Prenatal treatment

At 14 days of gestation pregnant rats received single injections of saline (controls), ara-C (50 mg/kg), ara-A (50 mg/kg) or ara-A (1 g/kg). All litters were born at either 21 or 22 days of gestation. Irrespective of the length of gestation litters were reduced to 8 animals (equal numbers of each sex) on day 22. At the same time a number of excess newborn were killed from each litter. No offspring of mothers receiving ara-A (1 g/kg) were examined beyond birth. In litters of the other 3 groups, up to 6 rats (2 males, 4 females) were killed at 25 days of age. From each control or ara-C (50 mg/kg) litter 2 male rats were weaned (at 25 days) and finally killed, after T-maze testing when 15 weeks old.

Postnatal treatment – single doses

Litters were reduced to 6 males at birth. At 5 days of postnatal age 2 rats (controls) in each of 6 litters were injected with saline while the remaining 4 rats in each litter were injected once with one of the following: ara-C (50 mg/kg), ara-C (250 mg/kg), ara-A (50 mg/kg) or ara-A (250 mg/kg). All animals were killed when 25 days old.

Postnatal treatment – multiple doses of adenine arabinoside

Litters were reduced to 6 males at birth. On days 3, 4, 5 and 7, two rats (controls) in each of 6 litters were injected with saline, 2 rats received ara-A (250 mg/kg on each day) and 2 rats received ara-A (1 g/kg on each day). All animals were killed when 25 days old.

Chemical assays

Brain DNA was determined according to Zamenhof, Bursztyn, Rich & Zamenhof (1964), and brain protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

Pigmentation

Pigmentation of these black and white hooded rats is affected by inhibitors of DNA synthesis, presumably when administration coincides with the elaboration of melanocytes. A pigmentation index was therefore determined at 25 days of age. This represented the proportion of the back (behind the base of the skull and to halfway down the flanks) which was black. The index was estimated by drawing, on graph paper, the approximate shape of the pigmented areas on a rat-shaped template.

T-maze

Rats aged 15 weeks were tested in a water T-maze as previously described for guinea-pigs (Adlard, Moon & Smart, 1974). The light cycle was 12 h red, beginning at 12 h 00 min, and 12 h white. Rats were required to swim and make a left-right discrimination in order to escape. Escape was possible from one arm only. An entry into a blocked arm, or re-entry into the start arm, constituted an error. Testing was carried out at 09 h 00 min-12 h 00 minutes. Each animal was given 10 trials per day for 6 days. On days 1-4 escape was possible from the same arm (left or right, randomly assigned to each rat). On days 5 and 6 the escape route was reversed and the ability to learn the reversal was examined.

Statistics

Except where stated otherwise mean values were compared by Student's *t* test.

Results

Prenatal growth after prenatal treatment with cytosine arabinoside or adenine arabinoside

A single dose of ara-C (50 mg/kg) at 14 days of gestation resulted in a birth weight deficit of 14% (Table 1). The brain was relatively more affected, with a weight deficit of 17%, and the brain/body ratio was significantly reduced. Treatment with ara-A, even at 1 g/kg, did not affect prenatal growth.

The brain weight deficit in offspring of ara-C

Table 1 Effect of single intraperitoneal dose of cytosine arabinoside (ara-C) or adenine arabinoside (ara-A) on day 14 of gestation on mean body and brain weight at birth.

	Control	*Ara-C (50 mg/kg)*	*Ara-A (50 mg/kg)*	*Ara-A (1 g/kg)*
Body wt. (g)	5.76 ± 0.79 (78)	4.98 ± 0.45* (60)	5.54 ± 0.64 (67)	5.56 ± 0.70 (68)
Brain wt. (mg)	228 ± 24 (31)	189 ± 20* (14)	236 ± 18 (27)	233 ± 18 (15)
Brain wt./body wt. ratio (%)	4.21 ± 0.26 (31)	3.82 ± 0.31* (14)	4.35 ± 0.40 (27)	4.38 ± 0.46 (15)

Results (mean ± s.d. of the number of animals indicated in parentheses) are based on the following numbers of litters: control, 7; ara-C (50 mg/kg), 6; ara-A (50 mg/kg), 5; ara-A (1 g/kg), 7.
* $P < 0.001$, compared with controls.

treated mothers reflected a deficit in number of brain cells as assessed by total DNA (Table 2). Brain protein concentration was also reduced, but the protein/DNA ratio (Table 2) was normal, suggesting that average cell size was not significantly affected.

Table 2 Effect of a single intraperitoneal dose of cytosine arabinoside (ara-C) on day 14 of gestation on mean brain DNA and protein at birth.

	Control	*Ara-C (50 mg/kg)*
Number of rats	31	14
Total brain DNA (μmol DNA-P)	1.65 ± 0.20	1.17 ± 0.12*
Brain protein (mg/g wet wt.)	67.7 ± 6.9	62.1 ± 5.8†
Brain protein (mg/μmol DNA-P)	9.51 ± 2.28	10.04 ± 1.39

Results (mean ± s.d.) are based on 7 control and 6 ara-C-treated litters.
* $P < 0.001$, † $P < 0.02$, compared with controls.

Postnatal treatment with cytosine arabinoside

A linear regression analysis shows that a single dose of ara-C at 5 days of age significantly reduced cerebellar weight and the ratio between cerebellar weight and brain weight at 25 days (Table 3). Although whole brain weight following the higher dose of ara-C was significantly less than that in controls ($P < 0.02$), this is entirely attributable to the effect on cerebellar weight. There was no effect on body growth. In ara-C treated animals hair growth was delayed by several days, but the extent of pigmentation of the hair was not different from that of the controls (compared with results on animals receiving ara-C prenatally).

Postnatal treatment with adenine arabinoside

A single dose of ara-A at 5 days of age did not affect growth of the body, whole brain or cerebellum to 25 days of age (Table 3). Half of the rats receiving 4 daily doses of ara-A (1 g/kg) failed to thrive and died before 25 days (Table 4). This represented a significant ($P < 0.01$, Fisher test) mortality compared with control or ara-A (4 doses

Table 3 Effect of a single intraperitoneal dose of cytosine arabinoside (ara-C) or adenine arabinoside (ara-A) at 5 days of postnatal age on mean body and brain weight at 25 days of age.

	Control	*Ara-C (50 mg/kg)*	*Ara-C (250 mg/kg)*	*Ara-A (50 mg/kg)*	*Ara-A (250 mg/kg)*
Number of rats	11	6	7	7	6
Body wt. (g)	55.2 ± 10.1	53.3 ± 14.4	48.3 ± 10.4	53.9 ± 12.7	57.2 ± 12.1
Brain wt. (mg)	1387 ± 110	1370 ± 106	1280 ± 111	1371 ± 91	1392 ± 132
'Forebrain' + 'Stem' (mg)	1188 ± 86	1192 ± 88	1128 ± 85	1177 ± 73	1193 ± 100
Cerebellum wt. (mg)	195 ± 24	179 ± 15	152 ± 26	194 ± 22	199 ± 31
Cerebellum wt./whole brain wt. ratio (%)	14.0 ± 1.0	13.0 ± 0.5	11.8 ± 1.1	14.1 ± 0.8	14.2 ± 0.9

Results (mean ±s.d.) are based on 6 litters.
Linear regression analysis of effects of ara-C within this dose range on cerebellum weight and cerebellum weight/whole brain weight ratio shows that both are highly significant ($P < 0.001$).

of 250 mg/kg) animals, none of whom died. There was a decrease (Table 4) in cerebellar weight (10%) in the 4 x 1 g/kg group but this was rather small compared with the reduction (22%) observed after one sixteenth the quantity of ara-C (single dose of 250 mg, Table 3). Postnatal treatment with ara-A affected neither growth of the hair nor its pigmentation.

Postnatal growth after prenatal treatment with cytosine arabinoside or adenine arabinoside

A single dose of ara-C (50 mg/kg) at 14 days of gestation resulted, at 25 days of postnatal age, in a 22% deficit in brain weight, but with no significant effect on body weight (Table 5). Cerebellar weight was affected less than that of whole brain. Prenatal treatment with ara-A (50 mg/kg) had no effect on postnatal brain growth.

Adult (15-week-old) male rats whose mothers received ara-C (50 mg/kg) prenatally also showed specific microcephaly in that body weight was normal, but brain weight was reduced by 15% (Table 6).

Table 4 Effect of daily intraperitoneal doses of adenine arabinoside (ara-A) on postnatal days 3, 4, 5 and 7 on mean body and brain weight at 25 days of age.

	Control	*Ara-A (4 x 250mg/kg)*	*Ara-A (4 x 1g/kg)*
Number of rats	11	12	6
Body wt. (g)	60.1 ± 7.3	60.8 ± 5.9	53.2 ± 4.5*
Brain wt. (mg)	1402 ± 59	1405 ± 43	1361 ± 62
Cerebellum wt. (mg)	199 ± 11	199 ± 11	182 ± 14*
Cerebellum wt./whole brain wt. ratio (%)	14.2 ± 0.5	14.2 ± 0.6	13.4 ± 0.7*

Results (mean ± s.d.) are based on 6 litters.
* $P < 0.05$, compared with controls.

Table 5 Effect of a single intraperitoneal dose of cytosine arabinoside (ara-C) on day 14 of gestation on mean body and brain weights at 25 days of age, and on hair pigmentation.

	Control	*Ara-C (50 mg/kg)*	*Ara-A (50 mg/kg)*
Number of rats	39	23	28
Body wt. (g)	55.1 ± 7.7	51.6 ± 7.7	57.1 ± 3.8
Brain wt. (mg)	1375 ± 67	1069 ± 107*	1385 ± 40
Cerebellum wt. (mg)	189 ± 12	169 ± 19*	196 ± 18
Brain wt./body wt. ratio (%)	2.50 ± 0.27	2.10 ± 0.21*	2.43 ± 0.26
Cerebellum wt./whole brain wt. ratio (%)	13.7 ± 0.5	15.9 ± 1.7*	14.1 ± 0.8
Pigmentation index (%)	30.3 ± 6.0	14.5 ± 4.8*	31.0 ± 3.5

Results (mean ± s.d.) are based on the following numbers of litters: control, 7; ara-C, 6; ara-A, 5.
* $P < 0.001$, compared with controls.

Pigmentation

Ara-C (50 mg/kg) at 14 days of gestation severely reduced the formation of hair pigment in offspring (Table 5). Figure 1 shows two typical control adults compared with two animals of the ara-C group. The latter show normal head pigmentation, but their backs have very little pigment. Ara-A had no effect on pigmentation (Table 5).

T-maze

During the first four days of testing in the T-maze the control rats improved progressively in terms of both errors made and number of errorless runs (Figure 2). In contrast, those animals treated prenatally with ara-C did not improve after day 2. Ara-C rats made significantly more errors than controls on days 3 and 4, and fewer errorless runs on day 4 (Figure 2). Over days 1-4, the ara-C group made 80% more total errors than the controls (Table 6). Despite their relatively poor performance on initial learning, ara-C rats did not differ from controls on reversal (days 5 and 6, results not shown). Behavioural testing was not carried out on rats treated prenatally with ara-A, since this treatment had no effect on later brain growth.

Table 6 Effect of a single intraperitoneal dose of cytosine arabinoside (ara-C) on day 14 of gestation on mean body and brain weights and on T-maze errors by 15-week-old male rats.

	Control	*Ara-C (50 mg/kg)*
Number of rats	12	11
Body wt. (g)	304.± 35	294 ± 32
Brain wt. (mg)	1760 ± 56	1497 ± 68*
Brain wt./body wt. ratio (%)	0.584 ± 0.062	0.512 ± 0.045†
Total T-maze errors (days 1-4)	6.1 ± 3.6	10.9 ± 3.9†

Results (mean ± s.d.) are based on 6 litters of each group.
* $P < 0.001$, † $P < 0.01$, compared with controls.

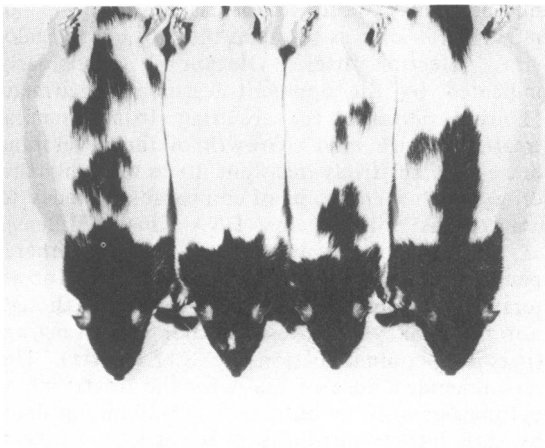

Figure 1 Hair pigmentation in 15-week-old male rats. Each rat was from a different litter. Animals at far left and far right were controls. The two centre rats were offspring of mothers receiving cytosine arabinoside (50 mg/kg) on day 14 of pregnancy.

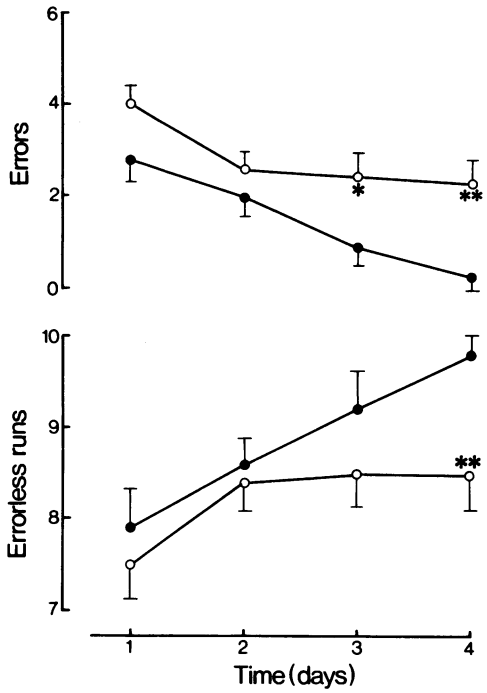

Figure 2 T-maze performance by adult male rats. Results represent means (± s.e. mean) of 12 control rats (●), and of 11 cytosine arabinoside (ara-C) rats (○) whose mothers received ara-C (50 mg/kg) on day 14 of gestation. Each animal was given 10 runs on each day of testing.
* $P < 0.05$, ** $P < 0.01$, compared with controls.

Discussion

In attempts to treat cytomegalovirus infection in infants ara-C has been administered over about 10 days in a total dose of approximately 50 mg/kg (Plotkin, 1972). This is a similar regime to that used in leukaemia and; in the present work, the same dose was given, but as a single injection. Species extrapolations are particularly difficult in the present context because of the very rapid rate of brain development in the rat. At this stage of brain development, a day in the life of a rat is equivalent to more than a month in the life of a human child. Thus it may be that even a single injection in the rat exposes the developing brain to the drug for a relatively longer period than multiple daily dose in man. For these reasons the present results are probable pointers to the qualitative, but not the quantitative, effects of ara-C on human brain development.

Ara-C exerted the much more severe effect on brain growth when given prenatally, at a time of initiation of rapid neuronal multiplication (Croskerry *et al.*, 1973). The deficit in brain cells at birth must have been largely of neurones, since prenatal brain cell division in this species is predominantly neuronal. For this same reason it was also probably permanent. Similarly glial cell multiplication in the rat brain is almost exclusively postnatal and therefore it is this process which must have been inhibited after ara-C treatment at 5 postnatal days of age. This had no effect on whole brain growth which could not be accounted for by the effect on the cerebellum.

From this it follows that neuronal (in contrast to total 'brain cell') number may be an important determinant of mature brain size, and the results on cerebellar growth support this concept. Neurogenesis in the rat cerebellum, unlike that in the rest of the brain, is primarily a postnatal event. Correspondingly, cerebellar growth was most retarded, both absolutely and relative to whole brain, when ara-C was given postnatally. The effects of ara-A, ara-C and hypoxanthine arabinoside on the developing cerebellum have been reported recently in considerable detail (Fishaut, Connor & Lampert, 1974).

The effects of prenatal ara-C in many ways resembled those observed after prenatal treatment with hydroxyurea (Adlard & Dobbing, 1975). Neither ara-C nor hydroxyurea produced any major malformations, but both treatments reduced the extent of hair pigmentation, probably through interference with melanoblast division and/or

migration. Ara-C seemed to produce a more specific effect on the brain than hydroxyurea in that microcephaly (low brain/body ratio) was observed as early as the time of birth. Moreover, the stunting of postnatal bodily growth, observed after hydroxyurea, was not found in rats treated with ara-C.

Ara-A was not toxic to prenatal brain development, even at 20 times that dose of ara-C which produced a 29% deficit in brain DNA at birth (Tables 1 and 2). In addition, prenatal ara-A did not affect postnatal brain growth. Postnatal treatment with ara-A had a small effect on cerebellar growth, but only at very high and multiple doses. Since these doses approached the LD_{50} observed in adult mice (Kurtz *et al.*, 1969) and were associated with a high mortality and impaired bodily growth, the effect on the cerebellum may well have resulted from general debility rather than from a specific influence on the brain.

The present results suggest that ara-C may be unsuitable as an antiviral agent for administration at a time of major brain development. This would apply particularly if the drug were given in early or mid-pregnancy where its effects on brain growth might be at least as severe as those of cytomegalovirus infection itself. Caution is particularly indicated by the apparent learning impairment (T-maze performance) resulting from prenatal treatment with ara-C. Growth of the developing brain was relatively resistant to ara-A, and this drug, which seems to be of comparable potency to ara-C in its action against DNA viruses (Miller *et al.*, 1969), may be more acceptable as a therapeutic agent. Nevertheless ara-A has a known teratogenic potential in developing rabbits (though not in monkeys) at doses greater than 3 mg/kg, (personal communication by S.M. Kurtz). The recommended dose of ara-A for the treatment of cytomegalovirus in humans is 15-20 mg/kg daily by slow intravenous infusion for at least 10 days. Even ara-A should therefore probably be avoided during pregnancy.

We thank Dr J.A.L. Gorringe of Parke, Davis and Co. for advice and a gift of adenine arabinoside. This work was supported by the Medical Research Council and the National Fund for Research into Crippling Diseases.

References

ADLARD, B.P.F. & DOBBING, J. (1975). Maze learning by adult rats after inhibition of neuronal multiplication in utero. *Pediat. Res.* (in press).

ADLARD, B.P.F., MOON, S. & SMART, J.L. (1974). Discrimination learning in ascorbic acid-deficient guinea-pigs. *Nature, Lond.*, **247**, 398.

BERENBERG, W. & NANKERVIS, G. (1970). Long-term follow-up of cytomegalic inclusion disease of infancy. *Pediatrics, Springfield*, **46**, 403-410.

CROSKERRY, P.G., SMITH, G.K., SHEPARD, B.J. & FREEMAN, K.B. (1973). Perinatal brain DNA in the normal and growth hormone-treated rat. *Brain Res.*, **52**, 413-418.

DOBBING, J. & SANDS, J. (1973). Quantitative growth and development of human brain. *Archs Dis. Childh.*, **48**, 757-767.

FISHAUT, J.M., CONNOR, J.D. & LAMPERT, P.W. (1974). Comparative effects of arabinosyl nucleotides upon the postnatal growth and development of the rat. *Pediat. Res.* (in press).

HANSHAW, J.B. (1966). Cytomegalovirus complement-fixing antibody in microcephaly. *New Eng. J. Med.*, **275**, 476-479.

KRAYBILL, E.N., SEVER, J.L., AVERY, G.B. & MOVASSAGHI, N. (1972). Experimental use of cytosine arabinoside in congenital cytomegalovirus infection. *J. Pediat.*, **80**, 485-487.

KURTZ, S.M., FISKEN, R.A., KAUMP, D.H. & SCHARDEIN, J.L. (1969). Toxicity of 9-β-D-arabinofuranosyladenine in mice and rabbits. In *Antimicrobial Agents and Chemotherapy – 1968*, ed. Hobbs, G.L., pp. 180-189. Bethesda, Maryland: American Society for Microbiology.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**, 265-275.

McCRACKEN, G.H., Jr. & LUBY, J.P. (1972). Cytosine arabinoside in the treatment of congenital cytomegalic inclusion disease. *J. Pediat.*, **80**, 488-493.

McCRACKEN, G.H., Jr., SHINEFIELD, H.R., COBB, K., RAUSEN, A.R., DISCHE, M.R. & EICHENWALD, H.F. (1969). Congenital cytomegalic inclusion disease. A longitudinal study of 20 patients. *Am. J. Dis. Child.*, **117**, 522-539.

MILLER, F.A., DIXON, G.J., EHRLICH, J., SLOAN, B.J. & McLEAN, I.W., Jr. (1969). Antiviral activity of 9-β-D-arabinofuranosyladenine. I. Cell culture studies. In *Antimicrobial Agents and Chemotherapy – 1968*, ed. Hobbs, G.L., pp. 136-147. Bethesda, Maryland: American Society for Microbiology.

PLOTKIN, S.A. (1972). Cytosine arabinoside in the treatment of congenital cytomegalic inclusion disease. Editorial comment. *J. Pediat.*, **80**, 493-495.

SCHABEL, F.M., Jr. (1970). Purine and pyrimidine nucleosides as antiviral agents – recent developments. *Ann. N.Y. Acad. Sci.*, **173**, 215-220.

SIDWELL, R.W., ARNETT, G. & BROCKMAN, R.W. (1970). Comparison of the anticytomegalovirus activity by a group of pyrimidine analogs. *Ann. N.Y. Acad. Sci.*, **173**, 592-602.

STERN, H., ELEK, S.D., BOOTH, J.C. & FLECK, D.G.

(1969). Microbial causes of mental retardation. The role of prenatal infections with cytomegalovirus, rubella virus and toxoplasma. *Lancet*, **ii**, 443-448.

ZAMENHOF, S., BURSZTYN, H., RICH, K. & ZAMENHOF, P. (1964). The determination of deoxyribonucleic acid and of cell number in brain. *J. Neurochem.*, **11**, 505-509.

(Received October 21, 1974.
Revised January 31, 1975)