Biochemical Pharmacology, Vol. 17, pp. 1689–1697. Pergamon Press. 1968. Printed in Great Britain

THE INFLUENCE OF 6-MERCAPTOPURINE ON RAT PLACENTA AND FETUS*

J. ROBERT BRAGONIER† and MICHAEL J. CARVER

Departments of Biochemistry, Obstetrics, Gynecology and Psychiatry, University of Nebraska College of Medicine, Omaha, Nebr., U.S.A.

(*Received* 9 *November* 1967; *accepted* 9 *February* 1968)

Abstract—Forty-eight hr after an injection of a teratogenic dose of 6-mercaptopurine into maternal rats (day 12 of gestation), total body fetal DNA content was decreased by 20 per cent. No evidence of gross malformation, fetal or placental weight decrease, or edema could be detected at this stage of development. Within 6 hr after 6-mercaptopurine administration, decreases in incorporation of radioactive glycine into adenine and guanine of fetal and placental DNA and RNA could be demonstrated. Only the fetal RNA guanine fraction failed to exhibit a statistically significant decrease in glycine incorporation.

In fetuses and placentas 6 hr after injection, 6-mercaptopurine did not significantly alter incorporation of radioactive glycine into protein, or guanine to adenine molar base ratios. However, experimental fetal/placental ratios with respect to fresh pooled weight, guanine and adenine from DNA and guanine from RNA were decreased in comparison to control values.

Investigations on the effects *in vitro* of 6-mercaptopurine ribonucleotide on ribosylamine-5-phosphate pyrophosphate phosphoribosyltransferase from fetus and placenta indicated that both enzyme preparations were inhibited by the ribonucleotide.

THE PROBLEM of environmentally produced congenital malformations has come to the foreground in recent years. Most of the studies, however, have thus far been primarily descriptive; mechanisms by which external factors produce malformations are still largely unknown.

The present study, therefore, was undertaken to evaluate the effects of 6-mercaptopurine (6-MP), a known teratogen in rats,[1, 2] on various aspects of nucleic acid metabolism in the developing rat fetus and placenta. The results presented suggest that 6-MP acts on the developing tissues by interfering with the incorporation of radioactive glycine into RNA and DNA purines *in vivo* and by inhibiting *in vitro* the first enzyme specifically involved with the biosynthesis of purines, ribosylamine-5-phosphate: pyrophosphate phosphoribosyltransferase (EC 2.4.2.14).‡ It is not possible at this time, however, to suggest that a causal relationship exists between the teratogenicity of 6-MP and the effects reported.

* Supported in part by Grants Tl-HD-18 and HD-00370 from the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md.

† Present address: Department of Obstetrics and Gynecology Jefferson Medical College, Philadelphia, Pa.

‡ Abbreviation used, amidotransferase.

MATERIALS AND METHODS

Pregnant albino rats weighing 150–250 g were obtained from Holtzman Company, Madison, Wis. The day the animals were sperm positive was considered gestation day zero.

On gestation day 12, experimental animals were injected i.p. with a suspension of 6-MP (60 mg/kg). Because of its insolubility in water or salt solution at a physiological pH, the drug was suspended in carboxymethylcellulose (CMC); use of this inert vehicle minimized error caused by settling out of the drug. Controls received CMC alone.

For characterization of drug effect, the pregnant rats were sacrificed by cervical dislocation on gestation day 20 and the fetuses were delivered by cesarean section. Fetuses were examined closely for evidence of malformations or stillbirth and uteri, for resorption sites. Placentas and fetuses were blotted with filter paper, weighed on a Mettler semi-micro balance and quick-frozen prior to subsequent study.

For determination of fetal DNA, control and experimental pregnant rats were sacrificed on gestation day 14 and the fetuses examined for gross anomalies. Fetuses were individually homogenized in distilled water and a known volume of homogenate made 0·3 N with respect to trichloroacetic acid (TCA) by the addition of an equal volume of cold 0·6 N TCA. After chilling at 0° for 30 min, the protein and nucleic acid fraction was separated from the supernatant by centrifugation at 16,000 *g* for 30 min. The precipitate was extracted twice with 15-ml aliquots of 0·5 N perchloric acid at 70° for 15 min and the extracts were combined. DNA was determined on these extracts according to conventional methodology.[3, 4]

To determine the effect of 6-MP on *de novo* synthesis of nucleic acid purine, control and experimental pregnant rats received 50 μc of glycine-1- or -2-^{14}C in 1·0 ml of sterile normal saline i.p. on gestation day 12; experimental animals had been given a teratogenic dose of 6-MP (60 mg/kg) 1 hr previously. Five hr after receiving the isotope, rats were sacrificed and fetuses and placentas from each litter were pooled separately, blotted with filter paper, weighed and quick-frozen. At this time, control and experimental litters were paired on the basis of similar litter sizes and weights; all subsequent manipulations were made on pooled fetuses or placentas, a pair of litters at a time.

DNA and RNA were isolated, hydrolyzed and subjected to column chromatography;[5, 6] aliquots from the columns were monitored for radioactivity by a Nuclear-Chicago liquid scintillation spectrometer.

For the measurement of amidotransferase activity, fetuses and placentas in each litter were pooled separately and the enzyme was isolated according to McCollister *et al.*[7] The reaction mixture for the enzyme assay contained 150 μmole potassium phosphate buffer, (pH = 8·1); 0·12 μmole glutamine; 0·75 μmole phosphoribosyl-pyrophosphate (PPRP); 1·2 μmole of the 3-acetylpyridine analogue of nicotinamide adenine dinucleotide; 9·0 μmole $MgCl_2$ and 0·1 ml glutamic dehydrogenase (diluted 1 to 10 and dialyzed against 0·05 M potassium phosphate buffer, pH = 7·5); and sufficient enzyme fraction to contain 0·7 to 5·0 mg protein. Distilled water was added to make a total volume of 3·0 ml. Activity of the enzyme preparation was determined in a Beckman DU spectrophotometer at room temperature by measuring the increase in absorbance at 363 mμ during the period from 5 to 15 min after the reaction was initiated.[8] Protein content in all experiments was determined according to Lowry *et al.*[9]

The effect *in vitro* of 6-MP was determined on the amidotransferase by adding to the reaction mixture 6-MP in amounts ranging from 1·5 to 2·0 μmole. For studies on the effect *in vitro* of the ribonucleotide, the barium salt of 6-mercaptopurine ribonucleoside-5′-phosphate (6-MPRP; P.L. Biochemicals, Milwaukee, Wis.) was dissolved in an equimolar solution of $MgSO_4$, the resulting $BaSO_4$ was precipitated and varying amounts of the supernatant were added to the above reaction mixture to make it 0·2 to 2·0 mM with respect to 6-MPRP. For enzyme kinetic studies, the 6-MPRP concentration was maintained constant at 0·125 mM, while the concentrations of glutamine and PPRP were varied independently.

RESULTS AND DISCUSSION

Effect on deoxyribonucleic acid and purine synthesis

Comparison of stained skeletons of control and experimental fetuses revealed, in the latter, stunting of growth with retardation or absence of ossification of metacarpal and phalangeal centers and absence of one or two of the lower leg bones. Both radius and ulna, however, were invariably present. Marked branching and fusing of the ribs was noted and a degree of spina bifida was suggested by the divergence and irregularity of lateral processes of the lumbar and sacral vertebrae, with duplication of the central vertebral body.

Worthy of note was the consistency of the above findings. Although malformations varied to a small extent in severity (probably because of differences in absolute individual drug dosage between litters of different size and variations in fetal susceptibility within the litter), no unaffected fetuses were obtained from maternal animals receiving 6-MP at the teratogenic dose. At the other extreme, no increase in intrauterine death was apparent, as judged by unchanged average litter size and lack of increase in number of stillbirths or uterine resorption sites.

To determine whether malformation production by 6-MP is associated with an alteration in nucleic acid synthesis, total body fetal DNA content was measured on day 14 of gestation, 48 hr after the maternal rat had received a teratogenic dose of 6-MP. Experimental fetuses contained 20 per cent less DNA per unit weight than control fetuses (Table 1). At this state of development, affected fetuses demonstrated

TABLE 1. EFFECT OF 6-MP ON FETAL DNA CONTENT

	DNA (μg/100 mg fresh tissue)*
Control (8)	597 ± 27
Experimental (10)	479† ± 23

* Mean ± S.D.; No. of experiments is in parentheses.
† $P < 0.001$.

no evidence of gross malformation, anasarca or edema, and no decrease in weight from control values could be detected. Placentas similarly were normal in weight. In addition, dry/wet fetal weight ratios were the same in both groups.

To identify more precisely the site at which 6-MP disrupts nucleic acid synthesis, it was decided to study the synthesis of nucleic acid purine in fetal and placental tissues

during the time when 6-MP was present. This decision entailed working on the twelfth day of gestation with very small (and necessarily pooled) fetuses and placentas. Investigation within the first few hours after 6-MP injection was necessitated by the rapid and extensive metabolism of the drug.[10]

A decrease in incorporation of glycine-^{14}C into purines of fetus and placenta was observed after the administration of 6-MP (Fig. 1). Statistically significant decreases in

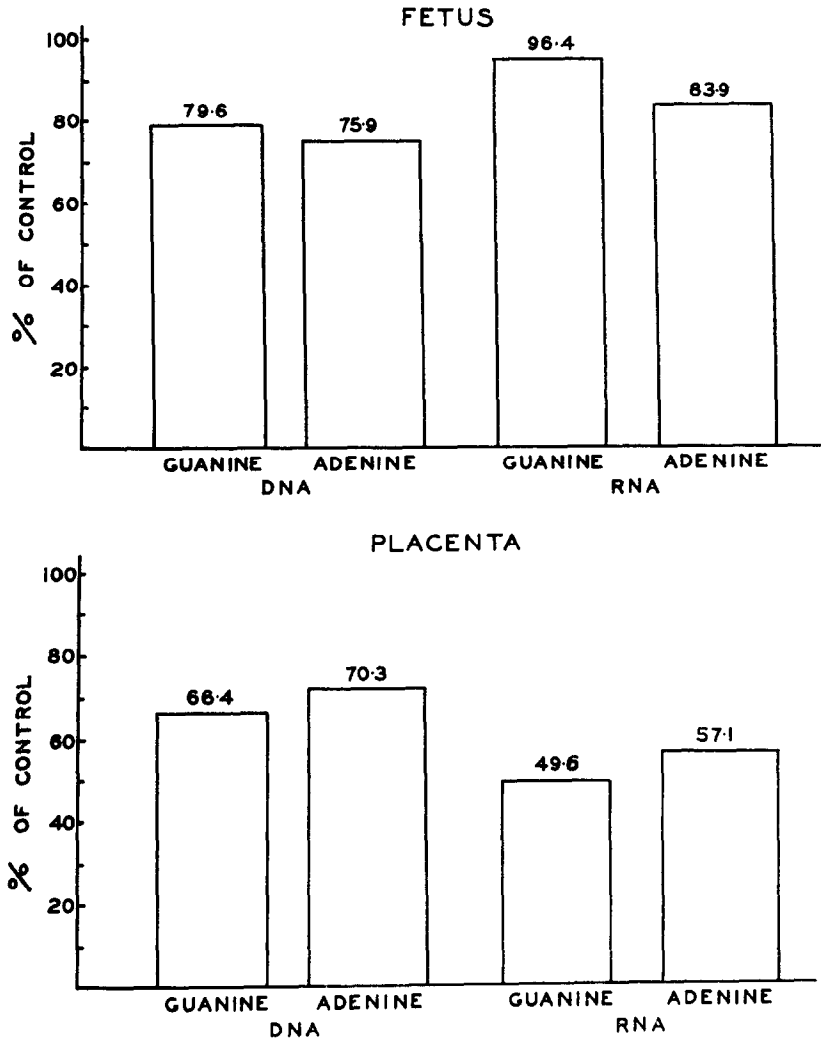

FIG. 1. Decreases in incorporation of radioglycine into experimental guanine and adenine fractions of fetal and placental DNA and RNA expressed as per cent of control. Data represent results obtained with 5 experimental and 5 control animals, with at least 6 fetuses pooled from each maternal animal. All purines studied are statistically different ($P < 0.01$) from the controls, with the exception of the fetal-guanine RNA group.

incorporation were documented for all purine fractions except the guanine fraction from fetal RNA. The reason for the sparing by 6-MP of this particular fraction is not immediately apparent.

Placental incorporation of glycine-^{14}C into purines was decreased to a greater extent than was fetal incorporation. This implies a proportionately greater inhibitory effect of 6-MP on placental than on fetal purine synthesis. This interpretation is in agreement with an earlier report[1] that 6-MP at a dose of 15 mg/kg, while producing no malformations, does cause a significant decrease in placental weight at term.

Six hr after injection, 6-MP did not significantly alter weights of pooled fetuses or placentas. As previously mentioned, even 48 hr was insufficient time for the effect of 6-MP on fetal and placental weight to be manifested. Incorporation of radioactive label from glycine-^{14}C into protein of fetuses and placentas was unchanged by 6-MP. The drug likewise had no measurable effect after 6 hr on purine content of fetuses and placentas per gram of fresh tissue, nor did it alter guanine/adenine molar base ratios.

In an attempt to gain additional information from accumulated data, the ratio of control purine to corresponding paired experimental purine for fetuses and placentas was calculated. From such calculations, it was noted that placental ratios were consistently lower than corresponding fetal ratios. A prediction was made that the fetal/placental ratio would, therefore, be decreased in experimental animals as compared with controls and the prediction was tested with regard to five parameters: fresh weight, guanine and adenine from DNA, and guanine and adenine from RNA (Table 2). Nonparametric analysis of the data indicated that differences in 4 of the 5 parameters examined were significant ($P < 0{\cdot}05$, one-tailed test).[11]

TABLE 2. EFFECT OF 6-MP ON FETAL/PLACENTAL RATIOS

Experiment No.	Fresh wt.	DNA guanine	DNA adenine	RNA guanine	RNA adenine
Control 1	0·44	0·39	0·37	0·48	0·50
Experimental 1	0·33	0·38	0·33	0·36	0·41
Control 2	0·43	0·35	0·37	0·23	0·23
Experimental 2	0·42	0·30	0·29	0·21	0·29
Control 3	0·35	0·38	0·42	0·37	0·37
Experimental 3	0·20	0·24	0·25	0·25	0·28
Control 4	0·41	0·38	0·33	0·48	0·42
Experimental 4	0·16	0·20	0·18	0·21	0·22
Control 5	1·02	0·84	0·74	0·95	0·95
Experimental 5	0·28	0·30	0·31	0·40	0·37

These data suggest that within 6 hr after 6-MP administration, the fetus demonstrates a more marked retardation in weight and purine increase than does the placenta. An alternate explanation is that within 6 hr after the injection, placentas from experimental animals increase disproportionately in weight and purine content. This contention is not supported by the findings, previously stated, that the effect of 6-MP on the placenta is that of suppression of weight[1] and of incorporation of glycine into purine. The implication, then, is that the overall effect of 6-MP on the fetus, while less severe than that on the placenta, is nonetheless sufficient to produce sudden inhibition of fetal growth and purine content increase. The placenta is more responsive to the effects of 6-MP than the fetus and is sensitive to lower doses of the drug, but the effects are manifested later or are more gradual in onset.

Effect on amidotransferase

The addition of 6-MP to the amidotransferase assay system did not result in any inhibitory effects except at extremely high concentrations of the base. This agrees with

previous work[8] that suggested that 6-MP must be converted to its ribonucleotide to inhibit the amidotransferase isolated from pigeon liver. The authors interpreted this finding to mean that the enzyme fraction lacked pyrophosphorylase activity required for the conversion of the purine base to its corresponding nucleotide. This explanation seemed applicable to fetal and placental enzyme fractions as well.

When 6-MP ribonucleotide (6-MPRP) was added to the reaction mixture in different concentrations *in vitro*, definite inhibition of amidotransferase was observed (Fig. 2).

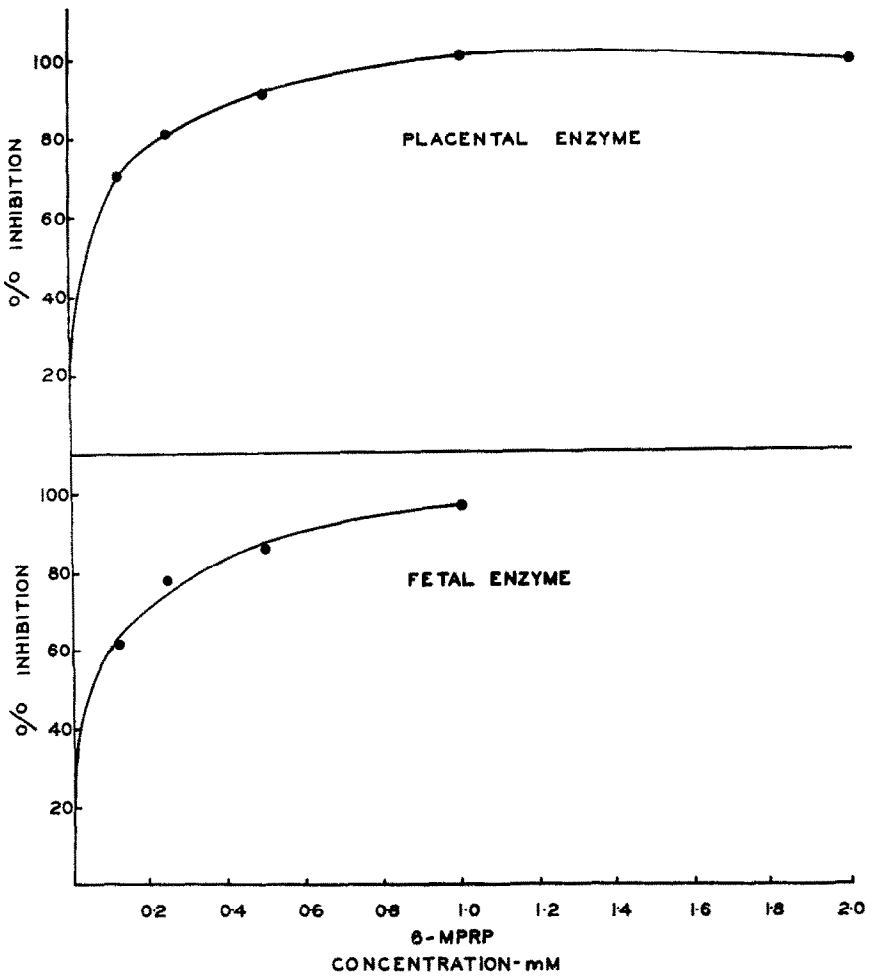

FIG. 2. Inhibition *in vitro* of placental and fetal amidotransferase activity by 6-mercaptopurine ribonucleotide.

Inhibition of both placental and fetal enzyme fractions approximated 100 per cent at a concentration of 6-MPRP equal to 1·0 mM.

The concentration of 6-MPRP was then maintained constant at 0·125 mM and concentrations of each of the two substrates, PPRP and glutamine, varied independently.[12] It is apparent that 6-MPRP behaves like a competitive inhibitor of

placental and fetal enzyme with respect to PPRP (Figs. 3 and 4). 6-MPRP may inhibit placental enzyme competitively with respect to glutamine, but the pattern of inhibition of fetal enzyme with respect to glutamine is similar to that referred to by McCollister *et al.* as mixed competitive-noncompetitive.[8] Furthermore, the observed inhibition was not due to inhibition of glutamic dehydrogenase, since only minimal inhibition was noted when this enzyme was studied separately.

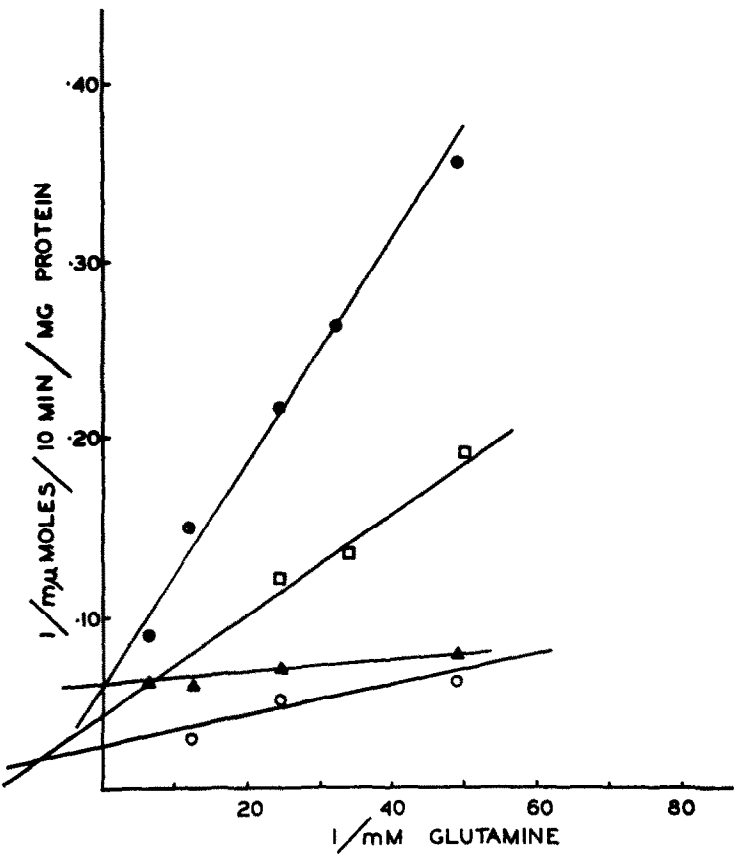

FIG. 3. Inhibition of the amidotransferase by 6-mercaptopurine ribonucleotide as a function of the concentration of glutamine. ●——●, Placenta enzyme with 6-MPRP (0·125 mM); ▲——▲, placental enzyme without 6-MPRP; □——□, fetal enzyme with 6-MPRP (0·125 mM); ○——○, fetal enzyme without 6-MPRP.

Caskey *et al.*[13] studied the effect of a combination of 6-amino- and 6-hydroxypurine ribonucleotides on the amidotransferase purified from pigeon liver and demonstrated inhibition greater than that which can be attributed to interaction of both inhibitors at a single site. From this evidence, they postulated the presence of at least two distinct inhibitor sites on the enzyme. Crude enzyme fractions were inhibited in addition by AMP, ADP, ATP, GMP, GDP and IMP; purification of the enzyme produced variable desensitization to inhibition by these nucleotides. However, when inhibition did occur, it was strictly competitive against PPRP and mixed competitive-noncompetitive against glutamine, in accord with findings previously mentioned.

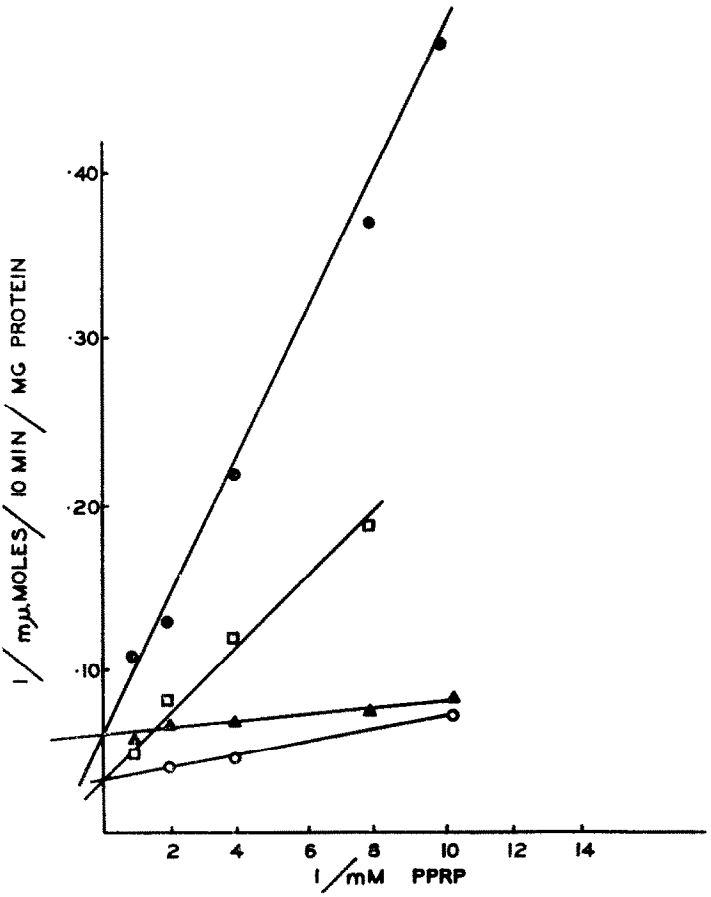

FIG. 4. Inhibition of the amidotransferase by 6-mercaptopurine ribonucleotide as a function of the concentration of PPRP. Symbols are the same as in Fig. 3.

Caution must always be used in drawing conclusions *in vivo* on the basis of observations from experiments *in vitro*. The importance of amidotransferase activity to nucleic acid synthesis during the critical period of rapid cell division or organ differentiation is well recognized. It seems reasonable, therefore, to theorize that inhibition of the enzyme in tissues during this critical period might have a profound effect on development.

REFERENCES

1. J. R. BRAGONIER, N. ROESKY and M. J. CARVER, *Proc. Soc. exp. Biol. Med.* **116**, 685 (1964).
2. M. L. MURPHY, *in Ciba Fdn Symp.* (Ed. G. E. W. WOLSTENHOLME and C. M. O'CONNOR p. 78, Little, Brown, & Co., Boston (1960).
3. K. BURTON, *Biochem. J.* **62**, 315 (1956).
4. E. VOLKIN and W. E. COHN, in *Methods of Biochemical Analysis* (Ed. D. GLICK), vol. 1, p. 287 Interscience, New York (1954).
5. L. L. BENNETT, H. E. SKIPPER, C. C. STOCK and C. P. RHOADS, *Cancer Res.* **15**, 485 (1955).
6. W. E. COHN, in *Chromatography* (Ed. E. HEFTMANN), p. 554. Rheinhold, New York (1961).
7. R. J. MCCOLLISTER, W. R. GILBERT, D. ASHTON and J. B. WYNGAARDEN, *J. biol. Chem.* **193**, 265 (1964).

8. J. B. WYNGAARDEN, H. R. SILBERMAN and J. H. SADLER, *Ann. N.Y. Acad. Sci.* **75**, 45 (1958).
9. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
10. G. B. ELION, S. BIEBER and G. H. HITCHINGS, *Proc. Am. Ass. Cancer Res.* **1**, 13 (1954).
11. S. SIEGEL, *Non-parametric Statistics for the Behavioral Sciences*, p. 116. McGraw-Hill, New York (1960).
12. H. LINEWEAVER and D. BURK, *J. Am. chem. Soc.* **56**, 658 (1934).
13. C. T. CASKEY, D. M. ASHTON and J. B. WYNGAARDEN, *J. biol. Chem.* **239**, 2570 (1964).