8. R. K. GHOLSON, Y. NISHIZUKA, A. ICHIYAMA, H. KAWAI, S. NAKAMURA and O. HAYAISHI, *J. biol Chem.* **237**, PC 2043 (1962).
9. G. P. MATHUR, C. Y. NG and L. M. HENDERSON, *J. biol. Chem.* **239**, 2184 (1964).
10. S. NATELSON, J. B. PINCUS and J. K. LUGOVOY, *J. biol. Chem.* **175**, 745 (1948).

Biochemical Pharmacology, 1966, Vol. 15, pp. 2136–2138. Pergamon Press Ltd., Printed in Great Britain.

Relationship between the metabolic effects and the pregnancy-interrupting property of 6-azauridine in mice*

(*Received* 26 *August* 1966; *accepted* 22 *September* 1966)

IT HAS been observed previously that both 6-azauridine (AzUR)[1] and 6-azacytidine,[2] antagonists of pyrimidine synthesis *de novo*,[3,4] are capable of interrupting pregnancy in both mice and rats.[5–7] The antimetabolites have their optimal effect when administered soon after implantation of the fertilized ovum has occurred; a single dose of AzUR (500 mg/kg), without producing any toxic effects in the host, results in complete resorption of the embryos. During the second half of pregnancy, even repeated administration of AzUR does not produce consistently an interruption of pregnancy. In an attempt to understand the difference between the effects of AzUR upon the early and late stages of fetal development, we have compared the metabolic transformation and biochemical effects of AzUR in 6-day embryos and in 15-day fetuses of mice.

Animals. Pregnant albino mice were obtained from the Charles River Breeding Laboratories, Inc., North Wilmington, Mass.

Compounds. 6-^{14}C-Orotic acid hydrate (4·9 μc/μmole) and ^{14}C-carboxyl-orotic acid hydrate (35 μc/μmole) were obtained from New England Nuclear Corp., Boston, Mass. 4,5-^{14}C-6-Azauridine (3 μc/μmole) was obtained through the courtesy of Prof. F. Šorm and Dr. J. Škoda, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague.

Effect of AzUR *upon orotic acid-incorporation* in vivo *in the fetus.* Mice, in days 6 and 15 of pregnancy, were injected i.p. with 6-^{14}C-orotic acid (4 μc and 8 μc respectively), together with AzUR (500 mg/kg); control mice received 6-^{14}C-orotic acid and saline. After 2 hr, the mice were sacrificed by decapitation and the uteri were excised. After removing the embryos from the uteri, the tissue was homogenized in cold 5% trichloroacetic acid (TCA). The homogenate was centrifuged and the precipitate, after being washed with TCA four times, was extracted with ethanol:ether (3:1) until the supernatant fraction was clear. The nucleic acids were extracted by heating the precipitate with 5% TCA at 95° for 30 min. After subsequent centrifugation, the TCA was extracted from the supernatant fluid by repeated shaking with ether. The optical density (260 mμ) and the radioactivity of the supernatant fraction were measured. The results are expressed as counts/min per ml/OD_{10}.

Phosphorylation of AzUR *in the embryos* in vivo. One hour after the i.p. administration of 5 μc of 4,5-^{14}C-6-azauridine, the mice were sacrificed and the embryos removed. The tissues were homogenized in 0·4 N perchloric acid and centrifuged, and the supernatant material was then neutralized with 8 N KOH. The resulting supernatant fraction was applied to Whatman 1 filter paper, and the phosphorylated AzUR was separated; a mixture of *n*-butanol, glacial acetic acid and water (10:1:3) was used.

Preparation of particle-free supernatant material from embryos. The embryos or fetuses, aged 6 and 15 days, respectively, were homogenized in cold 0·15 M KCl. The homogenate was centrifuged at 105,000 *g* for 90 min, and the resulting supernatant fraction was used as a source of enzyme activity for the reaction described in the following paragraph.

* The investigation was supported in part by Research Grant C-2817-10 from the National Cancer Institute, and in part by other funds.

Effect of AzUR *upon orotidylate* (OMP)*-decarboxylase activity in particle-free supernatant fraction of mouse embryos and fetuses.* The activity of OMP-decarboxylase and the effect of added AzUR, in the system, were determined by measuring the amount of $^{14}CO_2$ released from ^{14}C-carboxyl orotidylate, as described by Handschumacher *et al.*[4] The reaction mixture, which was incubated at 37° for 30 min, consisted of 0·5 ml of tris-HCl buffer (pH 7·4, 0·05 M), 0·5 μmole of 5-phosphoribosyl-1-pyrophosphate (PRPP), 0·5 μmole of ^{14}C-carboxyl-orotic acid (1×10^6 counts/min), 0·3 ml of cell-free supernatant material, and 0·75 μmole of ATP; total volume, 1·5 ml.

Radioactivity was determined by placing paper chromatogram strips or samples of solution into a mixture of PPO:POPOP:ethanol:toluene† and analyzed with a Packard Tri-Carb liquid scintillation spectrometer.

Effect of AzUR *upon the utilization of orotic acid* in vivo. AzUR, after its conversion to the 5′-monophosphate (AzUR-5′P), inhibits the decarboxylation of orotidylic acid.[3] One hour after the i.p. injection of a tracer dose of 4,5-^{14}C-6-azauridine, 5 μc, chromatographic analyses of the nucelotide pool of the embryonic tissues revealed that approximately 70 per cent of the antimetabolite was in an active nucleotide form, at both stages of fetal development. In 6-day embryos, the level of AzUR-5′P approximated 2×10^{-5} μmole/mg (wet weight).

TABLE 1. EFFECT OF 6-AZAURIDINE (AZUR)* UPON THE INCORPORATION OF 6-^{14}C-OROTIC ACID† INTO NUCLEIC ACIDS *in vivo*

Treatment	counts/min/per/ ml/OD_{10}	% inhibition
6-Day embryos		
Control	513	
AzUR	101	80·3
Control	557	
AzUR	152	72·6
Control	361	
AzUR	94	74·0
15-Day fetuses		
Control	144	
AzUR	59	58·9
Control	597	
AzUR	87	85·4

* 500 mg/kg, i.p.

† On days 6 and 15 of pregnancy, 4 μc and 8 μc, respectively, were injected i.p., together with the AzUR; the animals were sacrificed 2 hr later.

The effects of AzUR upon the utilization of orotic acid *in vivo* are shown in Table 1; it is evident that the analog inhibits utilization markedly, and that the effects 2 hr after drug administration are similar at both days 6 and 15 of fetal development.

Effect of AzUR *upon the decarboxylation of orotic acid* in vitro. To obtain more precise information phosphorylation of AzUR and its effects upon the decarboxylation of orotidylate, a particle-free supernatant fraction from both 6-day and 15-day embryos has been studied. The data in Table 2 indicate that the specific activities of orotidylic acid decarboxylase, as well as the extent of the inhibitions of the enzyme by metabolites of AzUR, are similar during both stages of embryonic development.

The results indicate that the inhibitory effects of AzUR upon orotic acid metabolism are very similar in days 6 and 15 of fetal development. In each stage of pregnancy, AzUR interfered with orotic acid metabolism *in vivo*, and, as determined with the cell-free supernatant fraction, with the metabolism of orotic acid *in vitro*. The data also indicate that the activities of OMP-decarboxylase are comparable during the two periods of development. These observations suggest that the difference in the pregnancy-interrupting effect of AzUR reflects a difference in the sensitivity of embryonic and fetal tissues to inhibition of nucleic acid synthesis, rather than to a difference in the effects of the drug. The inhibition of nucleic acid synthesis by AzUR in embryonic tissue may be attributed not

only to its effect upon OMP-decarboxylase, however, but also to other actions. Thus, it has been observed that AzUR in the chick embryo is capable of interfering with the utilization of uridine, as well as with the assimilation of orotic acid.[8]

TABLE 2. EFFECT OF 6-AZAURIDINE (AZUR) UPON THE DECARBOXYLATION OF ^{14}C-CARBOXYL-OROTIC ACID IN PARTICLE-FREE SUPERNATANT FRACTIONS OF 6-DAY EMBRYOS AND 15-DAY FETUSES

Age of tissue	Enzyme activity* (mμmoles/mg)	Concentration of AzUR	
		5×10^{-5} M	5×10^{-4} M
		(%)	
6 days	12	86·9†	94·7
15 days	10	88·7	94·8

* Activity of orotidylate decarboxylase, expressed as mμmoles orotate decarboxylated per mg protein.

† Per cent inhibition of enzyme activity.

Other compounds, such as 6-thioguanine and 6-chloropurine,[9] and 5-azacytidine,[10] which interfere with nucleic acid synthesis by mechanisms other than that of AzUR, also are capable of interrupting pregnancy when administered during the early stage of fetal development. The fact that these agents, as well as AzUR, are essentially inactive during the late stages of fetal development indicates that sensitivity to inhibition of nucleic acid synthesis may change during the course of embryonic growth. The pregnancy-interrupting effect of these agents, as well as that of AzUR, theoretically could reflect an action exerted upon the trophoblast, with death of the fetus resulting therefrom; however, the pharmacological and histological findings in mice[11] are insufficient to permit a conclusion concerning the primary site of action of the drug. Although studies of the lethal effect of AzUR on chick embryos *in situ*[8] have suggested a direct embryotoxic effect of the antimetabolite, a selective injury to trophoblastic tissue cannot be excluded. It is difficult to determine at this time, therefore, whether the placental damage reported in humans given AzUR[12] is a direct effect of the drug.

Acknowledgements—The authors wish to thank Miss Ingrid Grove for her technical assistance.

Department of Pharmacology,
Yale University School of Medicine,
New Haven, Conn., U.S.A.

KAREL RAŠKA, JR.‡
MORRIS S. ZEDECK§
ARNOLD D. WELCH

† 100 mg of *p*-bis[2-(5-phenyloxazolyl)]-benzene (POPOP); 8 g of 2,5-diphenyloxazole (PPO); 600 ml absolute alcohol; and 1400 ml toluene.

‡ Supported by a Special Fellowship of The Commonwealth Fund. Permanent address: Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague.

§ Supported by Research Training Grant CRTY-5012-09 from the United States Public Health Service.

REFERENCES

1. J. ŠKODA, V. F. HESS and F. ŠORM, *Coll. Czech. chem. Commun.* **22**, 1330 (1957).
2. F. ŠORM, J. SMRT and V. ČERNĚCKIJ, *Experientia* **17**, 64 (1961).
3. R. E. HANDSCHUMACHER and C. A. PASTERNAK, *Biochim. biophys. Acta* **30**, 451 (1958).
4. R. E. HANDSCHUMACHER, J. ŠKODA and F. ŠORM, *Coll. Czech. chem. Commun.* **28**, 2983 (1963).
5. M. A. SANDERS, B. P. WIESNER and J. YUDKIN, *Nature, Lond.* **189**, 1015 (1961).
6. K. ČEREY, J. ELIS and H. RAŠKOVÁ, *Biochem. Pharmac.* **14**, 1549 (1965).
7. J. ELIS and H. RAŠKOVÁ, *J. Physiol., Paris* **56**, 559 (1964).
8. A. D. WELCH, J. ŠKODA and M. GUTOVÁ, to be published.
9. J. B. THIERSCH, *Acta Endocr., Kbh.* **23**, Supp. 28, 37 (1956).
10. M. SVATÁ, K. RAŠKA, JR., and F. ŠORM, *Experientia* **22**, 53 (1966).
11. H. VORHERR and A. D. WELCH, to be published.
12. M. VOJTA and J. JIRÁSEK, *Clin. Pharmac. Ther.* **7**, 162 (1966).