ma 17-OHCS than found after hydrocortisone. In this respect, hydrocortisone cyclopentylpropionate was superior to other preparations studied. It is concluded that esterification alters the rate of steroid absorption.

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Received December 26, 1957. P.S.E.B.M., 1958, v97.

Teratogenic Changes in Early Chick Embryos Following Administration of Antitumor Agent. (Azaserine)* (23805)

RUSSELL J. BLATTNER, ALICE POLK WILLIAMSON AND LYDIA SIMONSEN
Department of Pediatrics, Baylor University College of Medicine, Houston, Texas

It has been previously reported that certain viruses, when inoculated in high concentration over the blastoderms of 48 hour chick embryos, produce specific teratogenic changes which are apparent on gross observation within 24 hours after inoculation(1-6). It has also been observed that antitumor agents often produce significant teratogenic changes in various embryonic organ systems. In recognition of the possible value of teratogenic studies in screening for antitumor agents, direct exposure of the frog embryo beginning at the 4-celled stage(7), and yolk sac inoculations of the chick embryo chiefly on fourth day(8), have been employed to determine teratogenic activity of various chemotherapeutic agents. The 2 methods, using different embryonic systems, however, have not always yielded comparable results for the same compound tested. It appeared that the 48-hour chick embryo, which consists of tissues in widely varying stages of proliferation with accompanying differentiation, might provide a sensitive testing system. If the changes should occur as rapidly as those following virus inoculations, this method would be particularly valuable in conserving time as well as laboratory space. Inoculations may be made directly over the cells of the developing blastoderm at this stage, thus avoiding possible interference by unknown variables such as absorption from the yolk sac, faulty access to susceptible organs via the circulatory system, or alteration of the compound *en route*. The substituted serine, O-diazoacetyl-L-serine (azaserine), having known antitumor activity (9), was selected for preliminary testing. This compound has previously been shown to produce developmental defects in the chick embryo following yolk sac inoculation at 4 days incubation(10), but produced less satisfactory results when inoculated into the yolk sac of unincubated eggs and eggs of 2 days incubation.

Materials and methods. The azaserine was obtained in pure form from National Institute of Health. As a control for specificity of action of the azaserine, experiments were also done using pure L-serine, obtained from Nutritional Biochemicals, Inc. For inoculations, the compounds were dissolved in saline buffered at pH 8.0. All materials used for inoculations were sterilized in routine manner except the compounds to be tested. The compounds were not sterilized because of the possibility of alteration by the process. Addition of antibiotics was omitted to avoid another variable. Broth cultures were made of all

* This investigation was supported by research grant from Nat. Cancer Inst., U.S.P.H.S.

inocula, and occasionally of embryos dying after inoculation, but little difficulty was encountered from contamination. Fertile eggs, incubated in forced draft incubator at 99°F for 48 hours, were prepared by removing a square flap of shell from the side. The intact shell membrane was then flooded with saline. A slit was made down the center of the membrane to facilitate removal with forceps, care being taken not to injure the transparent vitelline membrane beneath. The blastoderm floats naturally at the top, and gentle rocking of the egg brings it into center of opening. The inoculum in .05 ml amounts of saline containing the various dosages was dropped onto the vitelline membrane directly over the embryo lying in center of blastoderm. The opening in the egg shell was then rimmed with a paraffin-vaseline mixture (1:3) and sealed with a flamed cover slip. After inoculation the eggs were incubated in a horizontal position with sealed area uppermost. Such embryos were in stages of development from 10-14 according to the criteria of Hamburger and Hamilton(11). Eggs receiving inoculations of saline only (pH 8.0) were included with each experimental group to determine whether other nonspecific teratogenic influences were present. Embryos were thereafter examined daily *in vivo* at 24 power magnification or harvested at specified intervals. Embryos harvested at 5 days incubation or less were fixed in Bouin's solution, the picric acid staining later removed in lithium-carbonate-alcohol. Embryos were stained overnight in very dilute aqueous solution of alum cochineal, cleared and examined while still floating in the clearing fluid. General growth inhibition was estimated by visual comparison with control embryos. General developmental retardation was determined by comparison of developmental stages with those of saline controls. Embryos were tabulated as showing specific organ defects if organs in question were retarded beyond that of the estimated developmental stage of embryo being examined, or if they were obviously small in relation to adjacent structures, or obviously different in structure from that of any normal stage. In searching for a dose of azaserine which might produce gross teratogenic changes within a short time without being lethal to embryos, a series of diminishing doses of azaserine was prepared in saline and inoculated into respective groups of eggs of 48 hours incubation. The embryos, examined *in vivo* 24 hours later, showed changes in the treated embryos, ranging from severe in group receiving the largest dose, to no visible changes in group receiving the smallest dose. The most obvious gross disturbance consisted of varying degrees of stagnation of blood cells in the smaller vessels of the circulatory system and varying degrees of hemorrhage. The heart continued to beat rhythmically, but circulation of blood appeared markedly impaired. In such embryos the extraembryonic blastoderms were small in comparison to controls. The embryos themselves appeared significantly different from controls. Exact changes were difficult to evaluate *in vivo,* so for fear that death might be imminent, all groups were harvested at 24 hours postinoculation for examination at low power magnification.

TABLE I. Effect of Various Doses of Azaserine on 12 Chick Embryos per Series, Inoculated at 48 Hour Incubation and Harvested 24 Hours Later.

		Teratogenic changes observed		
Dose (mg)	Surviving at 24 hr	General growth inhibition	Mean stage of development	Surviving embryos with specific defects
				%
.2	6	moderate	16.2	4 66
.1	7	"	15.3	7 100
.05	11	slight	15.4	9 82
.025	9	"	16.0	8 89
.013	11	none	16.4	7 64
.007	11	"	17.2	0 0
saline	11	"	17.1	0 0

Results. From the results shown in Table I, it is clear that azaserine in dosages of 0.012 mg or more produced general growth inhibition, general developmental retardation, as well as defective development in specific organs of the embryo (Fig. 1, 2).

It was found that as little as 0.06 mg of azaserine caused agglutination and hemolysis *in vitro* at 37.5°C when dissolved in 1 ml of a 0.5% suspension of washed erythrocytes from 17-day-old chick embryos. In view of possibility that agglutination and hemolysis *in vivo* were responsible for circulatory dis-

turbances, it appeared that the teratogenic changes observed might be secondary to the ischemic conditions produced. Circulation in the chick embryo is not established before stage 12. Therefore, studies were made to determine the effect of azaserine upon the embryo prior to this stage. Embryos of 24 hours incubation were in various stages of development, but none were advanced beyond stage 7. Exact staging of such young embryos was difficult *in vivo,* but presumably some were still in the streak stage. It is known that mechanical injury to vitelline membrane during this stage results in abnormal development of the embryo. This may account for the rather high incidence of abnormal embryos among controls. Controls inoculated with saline and harvested 24 hours later yielded 9 abnormal out of 22 embryos inoculated. However, among embryos inoculated with 0.025 mg azaserine and harvested 24 hours later, 25 out of 27 showed general retardation and abnormal development. More specific evidence that azaserine exerts a direct inhibitory effect was found, however, among embryos inoculated at 48 hours incubation, when an occasional embryo escaped gross circulatory disturbances but still suffered definite growth inhibition, developmental retardation and specific defects.

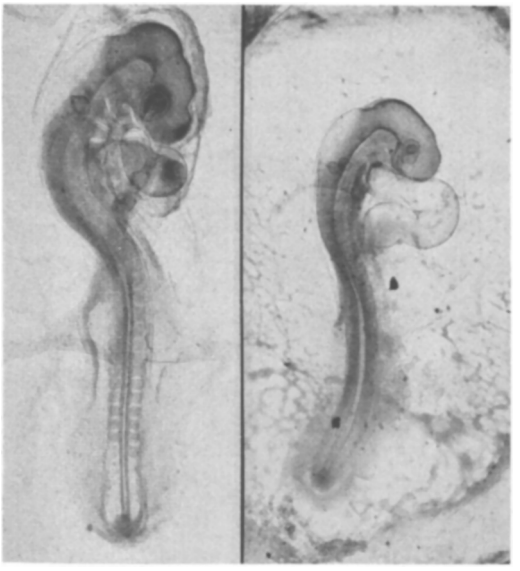

FIG. 1. Chick embryos of 72 hr incubation (×23). Left: Appearance of control embryo of avg size and development. Right: Appearance of embryo treated with azaserine 24 hr previously, showing characteristic growth inhibition and developmental retardation. Photographs by Visual Education Dept., Baylor Univ. College Medicine.

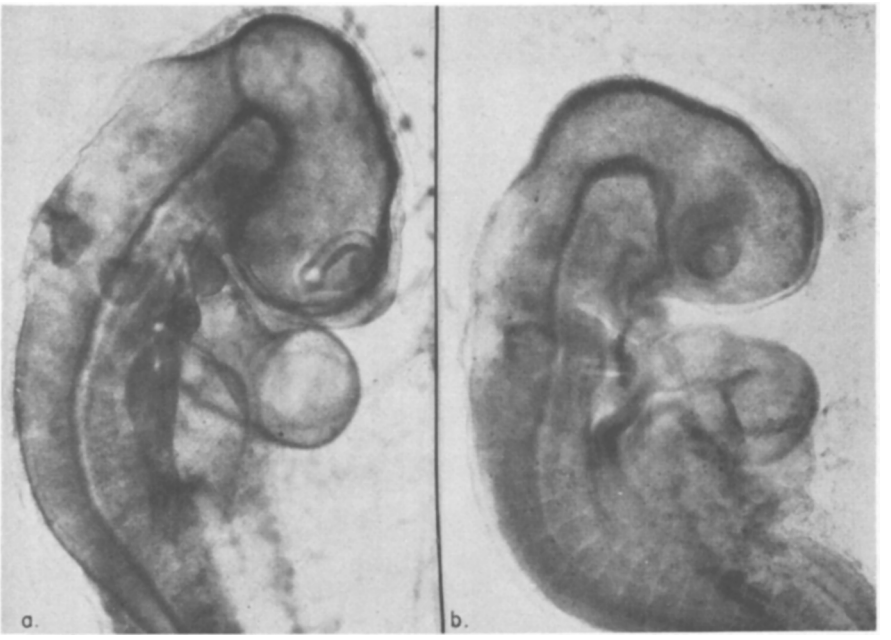

FIG. 2. Embryos of 72 hr incubation treated 24 hr previously with azaserine. a. Shows small lens. b. Shows retarded development of encephalon in region of forebrain and midbrain, including retarded development of optic cup and lens primordia.

TABLE II. Teratogenic Changes Observed in Embryos Harvested at Various Stages after Inoculation.

Inoculum	Harvest: Day after inoc.	Total eggs inoc.	Embryos surviving at harvest	No. surviving abnormal embryos	%	Observed effects in surviving embryos: Growth inhibition	Mean stage develop.	Retard. eneph.	Retard. optic cup	Small optic cup	Small lens	Small oto-cysts	Micromelia	Other
Aza.—.05-1.0 mg	1	87	49	35	73	Marked	14.5	12	30	0	12	1	*	
Control—saline	1	43	39	1	3	None	16.9	0	0	0	0	0	*	Flattened encephalon
Aza.—.05 mg	3	38	22	18	83	Marked	19.8	6	3	13	1	3	7	
Control—saline	3	10	10	0	0	None	25.5	0	0	0	0	0	0	
Aza.—.05 mg	5	37	16	11	70	Moderate	28.0	1	*	2	0	2	11	
Control—uninoc.	5	18	13	1	8	None	29.0	0	*	0	0	0	1	
Aza.—.05 mg	19	46	4	2	50	Marked		*	*	0	0	*	2	

* Stage at examination not conducive to gross observation of this defect.

To study survival of embryos as well as types of teratogenic changes found in embryos surviving various intervals after treatment with azaserine, groups of eggs were inoculated with 0.05-0.1 mg of the compound. Daily observation revealed that embryos with severe circulatory disturbances did not always die, but in many instances sustained re-establishment of circulation. Such embryos remained severely retarded and abnormal in development. From results shown in Table II, it is clear that teratogenic changes are seen in high percentage of surviving embryos up to time of hatching, although few reached this stage and none hatched. The encephalon, eye primordia and limb buds appeared more susceptible to inhibitory effects than did other tissues. The auditory vesicles were retarded rarely, but occasionally they showed unusually pointed appearance at site of attachment to body ectoderm. Exact staging was difficult in many embryos because the tissues tended to be blurred and took stain poorly. Some organs, particularly the auditory vesicles, were often seen to be developed beyond the stage assigned to the embryo through the staging criteria used. Since these organs were not developed beyond their counterparts in the control group, it was assumed they had escaped the inhibitory effect suffered by the rest of the embryo and represented more truly the stage the embryo should have attained. There was some shift in pattern of defects observed at different stages. However, certain defects are observed more readily at some stages than others. For instance, limb bud retardation can not be observed before stage 17, since limb buds are not sufficiently developed before this stage; and optic cups, after stage 19, have passed beyond the point where mild retardation of invagination can be observed grossly. Saline controls did not exhibit a significant number of abnormal embryos, and unsubstituted L-serine inoculated in doses of 0.2 and 0.05 mg failed to show any teratogenic changes when examined 1 day and 3 days after inoculation.

Discussion. Tissues during early embryonic life undergo proliferation at a greater rate than tissues of the fully developed individual, or even than tissues of the later em-

bryo. Furthermore, for each organ there are specific periods of increased proliferation at the time of beginning differentiation, presumably associated with altered metabolic activity. It is well known that tissues in such 'critical' states are more susceptible to the action of teratogenic agents than during less active periods. The chick embryo between stages 10 and 14 (approximately 48 hours incubation) is probably at the optimal stage for studies of teratogenic activity, using the technics described, since the inoculated material gains direct access to many organs in critical states of differentiation from the body ectoderm(2,3). Embryos younger than this do not show sufficient organ differentiation and do not survive the inoculation procedures as well. In embryos older than this the gross changes are not as striking, and the harvested embryos are more difficult to examine. It is interesting, therefore, that azaserine treatment of embryos at this critical period resulted in general retardation of growth and differentiation as well as specific retardation of organs which were in such critical stages of development, *i.e.* brain, optic primordia and limb buds. On the other hand, the auditory vesicles in similarly 'critical' stages were rarely affected. The reason for this is not known. However, organs undergoing differentiation doubtless differ significantly from each other, as well as from undifferentiating tissue.

Summary. The substituted serine O-diazoacetyl-L-serine (azaserine) was shown to produce teratogenic changes in the chick embryo when inoculated over the blastoderm at 48 hours of incubation. Such changes, observed on gross examination of harvested specimens within 24 hours after inoculation, consisted of general growth inhibition, general developmental retardation and specific retardation in the development of the encephalon, optic cup and lens vesicle. Examination of embryos allowed to continue incubation for longer periods of time revealed that teratogenic changes were present in a high percentage of the surviving embryos. Retardation of the encephalon was less apparent on gross examination of older embryos, but retardation of the limb buds was observed in high percentage of embryos examined from 3 days after inoculation up to the time of hatching. No gross changes were observed when control inoculations were made using saline or unsubstituted L-serine. The possible application of this technic in the study of antitumor agents was discussed.

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