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100-1000 **PHYSIOLOGICAL STUDIES ON CONGENITAL DEFORMITY**
ANALYSIS OF ACTION OF RADIATION AND RADIOMIMETIC TREATMENTS *3/7*

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INTRODUCTION

An extensive progress report was submitted last year. Part I of the 1967 report consists of copy of 2 abstracts submitted to the Teratology Society entitled "A Fetal Scoring System for Studying Syndrome Response to Teratogenic Treatments, Teratogenicity and Embryo Toxicity following X-Irradiation." Application of the recently developed scoring system will be illustrated by 4 tables showing interaction of X-irradiation with hypoxia (Table 1); comparisons of dual and single teratogenic treatments comparing X-irradiation with five other teratogenic treatments (Table 2); effect of type of mating and parity on teratogenicity (Table 3); and effect of commercial rations on teratogenicity (Table 4).

The second part of the progress report will summarize activities in conjunction with explanted embryos exposed to irradiation and derivatives of thalidomide.

Also included as a part of the report are manuscripts submitted for publication. "The chick embryo as a test system for teratogenic drugs" has been accepted for publication in a volume to be published by the Chicago Press. Another article entitled "Comparative Pharmacology in Relation to Teratogenesis" is being published in a forthcoming issue of the Federation Proceedings.

① see COO-1270-3

② see COO-1270-2

I. ANALYSES OF SYNDROME SCORES

A Fetal Scoring System for Studying a Syndrome Response to Teratogenic Treatments

Abstract for Teratology Society, 1967

Six teratogenic treatments applied to mice (strain 129) singly and in all possible combinations of dual treatments produced anomalies in a descending order of frequency (Table 1), viz., abnormal lumbar centra, abnormal thoracic centra, absent and fused ribs, costalization of the first lumbar vertebra, and cranioschisis. The following procedure was devised to compare experimental groups on the basis of a syndrome.

This procedure was applied only after it was observed by inspection that the proportions between thoracic centra, lumbar centra, costalized lumbar number one, abnormal ribs and cranioschisis were approximately evenly distributed among all experimental groups. The assumption, therefore, is that comparisons are made between the same syndrome of anomalies. The frequency of defects was obtained for all of 2,400 fetuses from 21 experimental groups as well as the frequency of corresponding defects among nontreated control animals. The control frequencies (fc), considered as background noise, were subtracted from the frequencies found among the treated animals (fe). Reciprocals of the noise-corrected values were adjusted (w) to make 100 the highest possible score. Thus, the defect having the highest frequency (lumbar centra) was weighted to give the smallest contribution, 10.6, while the defect having the smallest frequency (cranioschisis) contributed the greatest weight, 28.2.

$$w \frac{1}{fe_1 - fc_1} + w \frac{1}{fe_2 - fc_2} + \dots \dots \dots w \frac{1}{fe_n - fc_n} = \text{Syndrome Score.}$$

The syndrome score can be applied to the litter as a unit, by obtaining a ratio (syndrome-score-ratio) between the total value possible for a litter of a given size and the total of the observed syndrome scores. Comparisons of the scores for individuals and the score-ratios for litters indicate that among the 21 experimental groups, there seems to be close agreement between means ($\pm$ standard deviations) of litters when compared to means ($\pm$ standard deviations) of individuals. Weighted values derived from reciprocals of the observed frequencies obviate any problem arising from anomalies that may be associated with each other because this phenomenon was reflected in the survey of the population as a whole. Syndrome scores from experimental groups plotted as frequency histograms showed normal distribution curves.

The syndrome scores for individuals have enabled statistical analyses that seem more meaningful than summarizations of a single anomaly or complex tables summarizing multiple anomalies.

Table 1. Calculation of weighted values for anomalies derived from reciprocals of observed frequencies

	f_e	f_c	$f_e - f_c$	$\frac{1}{f_e - f_c}$	$w \frac{1}{f_e - f_c}$
	% abnormal in experimental animals	% abnormal in control animals	Frequencies corrected for background noise	Reciprocal	Adjusted Reciprocal
Lumber centrum #4	48.7	20.4	28.3	3.5	10.6
Thoracic centra 10-12	27.3	4.2	23.1	4.3	13.2
Costalized first lumbar	14.4	0	14.4	6.9	21.1
Abnormal ribs 5-7	11.4	0	11.4	8.8	26.9
Cranioschisis	11.3	0	11.3	8.9	28.2
			Syndrome Score		100.0

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Teratogenicity in the absence of embryo toxicity following X-irradiation.
(Abstract for the Teratology Society, 1967)

X-irradiation was administered to fasted and nonfasted mice of strain 129 during the ninth day of gestation in doses of 0, 100, 140 and 200 roentgens (Table 2). The eight experimental groups consisted of 89 to 177 fetuses collected on the 18th day post coitum. 100r given to nonfasted animals produced a syndrome score of 8.9. Young from fasted animals, in the absence of X-irradiation, produced a score of 11.9. Combined treatment, 100r plus fasting, produced a score of 21.2, the approximate sum of the first two. Similarly, fasting combined with 140r produced a score of 24.9, which is approximately the sum of 11.9 from fasting and 15.4 from 140r alone. If the fetal score of 2.9 that occurred as noise background in untreated controls is subtracted, the approximation to a summation effect comes very close. (Treatment with 200r produced a fetal abnormality score of 37 in both the fasted and nonfasted groups; no summation or synergistic effects were demonstrated.)

Toxicity (i.e. embryo and fetal death) was studied by observing frequencies of pregnancies, reabsorptions and implantation and numbers of live young. The frequency with which mated animals became pregnant varied between 60 and 86 percent for fasted and nonfasted animals receiving 0, 100 and 140 roentgens. The frequency of pregnancy was reduced to about half, 32.9 and 43.1, in both groups receiving 200r. The average number of implantations in those animals pregnant at 18 days post coitum varied between 5.9 and 8.2. No significant difference and no trend in number of implantations could be detected as a result of teratogenic treatments. The average number of resorptions per pregnant animal varied between 0.8 and 3.9. Among the nonfasted animals there was a trend of increasing number of resorptions correlated with the dose of irradiation. This trend was not confirmed among the fasted animals. Differences in rates of resorption between experimental groups were not significant.

The average number of live young at 18 days post coitum varied between 3.7 and 6.1. Differences between the eight groups were not significant, nor was there a demonstrable trend correlated with dose of irradiation.

The increasing frequencies of malformations were not accompanied by a reduction in the average number of implantations, resorptions or live young. It is concluded that teratogenic doses of X-irradiation were not toxic to embryos.

Table 2. Teratogenicity in relation to toxicity

	<u>Teratogenicity Syndrome Score</u>	<u>No. of Matings</u>	<u>Percent Pregnancy</u>	<u>Average No. Implantations</u>	<u>Average No. Resorptions</u>	<u>Average No. Live Young</u>
Nonfasted						
0r	2.9	23	86.9	5.9	0.8	5.2
100r	8.9	43	79.2	7.2	1.6	5.5
140r	15.4	29	75.9	7.3	2.0	5.3
200r	36.9	73	<u>32.9</u>	7.6	3.9	<u>3.7</u>
Fasted						
0r	11.9	52	60.8	6.7	2.3	4.4
100r	21.2	54	61.2	7.4	2.3	5.1
140r	24.9	26	80.7	8.2	2.1	6.1
200r	37.5	51	<u>43.1</u>	6.4	2.2	4.2

Supported by research grant AT(11-1)-1270 from the U.S. Atomic Energy Commission.

Note added to Progress Report:

The cleared and stained fetuses were observed for their developmental progress, i.e. stage of ossification. Animals treated with 200r showed about twice the frequency of odontoid processes nonossified, presphenoid bones nonossified and nonossification of thoracic centrum #1 than did the remaining experimental groups, thereby indicating that 200r resulted in retarded development. Since this retardation was associated with a high frequency of failure of pregnancies, and was not associated with toxicity, i.e., lethality of embryos, the data suggest that developmental retardation resulted from the effects of irradiation upon the mother.

Observations on developmental progress showed unexpected results when 140 roentgens were administered. The fetuses had a significantly higher frequency of ossified stapes, presence of odontoid process, presence of the presphenoid bone, ossification of thoracic centrum number one, and ossification of distal phalanges of the rear limb. Paradoxically, these fetuses with a malformation score of more than twice the untreated controls showed significantly more advanced maturation of the skeleton.

The data show that 200r of X-irradiation reduced the frequency of pregnancy without a significant effect on average number of implantations, reabsorptions or live young in the persisting pregnancies. The young, however, showed developmental retardation relative to ossification of stapes,

odontoid process, presphenoid bone, thoracic centrum #1 and distal phalanges of the rear foot. 140r of X-irradiation, although teratogenic, showed precocious maturation of the skeleton relative to stapes, odontoid process, presphenoid bone, thoracic centra and distal phalanges. Of the two teratogenic doses of X-irradiation, 200r showed retardation and 140r showed precocious maturation of the skeleton. These contrasting results should be confirmed.

X-irradiation Mitigates Teratogenicity of Hypoxia

When 100r were delivered early in the hypoxic period the teratogenicity syndrome score was increased from 20.2 to 30.2. These results are contrasted to X-irradiation when given late in the hypoxic period. Under this regimen the teratogenicity score was actually reduced from 20.2 to 9.0. Since hypoxia produced the larger syndrome score, it must be concluded that a teratogenic dose of X-irradiation mitigated the anticipated teratogenicity of hypoxia.

Table 3.

	Syndrome Score <u>$\pm$ standard deviation</u>	No. of <u>fetuses</u>
X-irradiation 100r	8.9 $\pm$ 9.4	(177)
X-irradiation 100r Late in hypoxic period	9.0 $\pm$ 11.6	(105)
X-irradiation 100r Early in hypoxic period	30.2 $\pm$ 20.5	(86)
Hypoxia	20.2 $\pm$ 22.2	(136)

Comparison of Dual and Single Teratogenic Treatments Mean Syndrome Score of Individuals ($\pm$ Standard Deviation)

X-irradiation more effectively sensitized embryos to increased probability of deformity than any of the other 5 teratogenic treatments. See Figures 4 and 6 (Runner, 1967e) for interpretation. Three of these 5 combinations approximated an additive effect; therefore, the data suggest that X-irradiation produced the same or similar syndrome of anomalies by unrelated metabolic pathways.

The number of dual treatments, 7 out of 15 combinations, that showed no interaction, nil effect, is unusual. These data suggest that the 7 teratogenic treatments affected separate steps in a shared enzymatic pathway.

Table 4.

Treatments	Iodoacetate	Hypoxia	Trypan Blue	Fast	9-Methyl Folic Acid	X-ray
X-ray	<14.1 ± 11.6*	>29.1 ± 24.3*	17.5 ± 9.1	>21.2 ± 20.1*	>14.3 ± 9.7*	8.9 ± 9.4
9-Methyl Folic Acid	<5.6 ± 9.9*	20.6 ± 21.9	<11.3 ± 11.4*	12.1 ± 12.1	9.1 ± 14.5	
Fast	29.4 ± 23.2	46.2 ± 26.3*	23.7 ± 23.7	11.9 ± 17.5		
Trypan Blue	<26.3 ± 22.5*	20.4 ± 18.5	18.3 ± 12.8			
Hypoxia	29.1 ± 20.7	20.2 ± 22.2				
Iodoacetate	35.8 ± 27.5					

*Dual treatment differs significantly, $P < 0.02$, from single treatment having the larger effect.

Factorial Experiment Showing Effect of Type of Mating and Parity on Teratogenicity. (Mean Syndrome-Score-Ratio for Litters)

Advanced parity increased the syndrome score ratio in fasted mice. This shows that embryos in older mothers appear more susceptible to malformations induced by 24 hours of fasting. Although relatively small difference in age did not affect the teratogenic syndrome score, the data do not permit distinction between age, per se and succession of pregnancy.

Matings induced with hormones increased the syndrome score ratio in nonfasted mice. If mice are any indication hormonal treatment leading to pregnancy increases the probability of malformations. These observations have important overtones in relation to the increasingly frequent use of post-menopausal hormones to induce pregnancy and multiple births in human beings.

Table 5.

<u>Mating Type</u>	<u>Mean No. of Ratio Litters</u>	<u>Parity</u>	<u>Mean No. of Ratio Litters</u>
<u>Nonfasted</u>			
Spontaneous	2.4 (43)	Nulliparous	3.3 (12)
		Uniparous	2.5 (16)
		Multiparous	3.0 (15)
Induced	<u>7.4</u> (33)	Nulliparous	3.5 (11)
		Uniparous	8.3 (12)
		Multiparous	10.3 (10)
<u>Fasted</u>			
Spontaneous	15.4 (57)	Nulliparous	11.0 (19)
		Uniparous	14.6 (22)
		Multiparous	<u>21.5</u> (16)
Induced	17.5 (33)	Nulliparous	17.2 (13)
		Uniparous	12.9 (11)
		Multiparous	<u>23.5</u> (9)

Interaction of Commercial Rations and Teratogenic Treatments. Mean Syndrome Score $\pm$ Standard Deviation (No. of Fetuses)

Interaction of Old Guilford diet with 9-methyl folic acid consistently sensitized embryos and provided significantly higher syndrome scores. The Old Guilford diet although not teratogenic in itself was a secondary factor that caused an increased syndrome score in all six experiments.

Table 6.

Purina Diet

Old Guilford Diet

*Chi square test gave P value < 0.02 .

II. BIOCHEMICAL STUDIES ON THE SILENT PERIOD IN TERATOGENESIS

A. X-irradiation

A year ago we reported results from chick embryos that had been irradiated with cesium 139 at the ten somite stage. Both the type and frequency of abnormalities in these chick embryos were reported at doses of 400, 600 and 800 roentgens.

Having observed the morphological consequences of irradiation, experiments during the past year have been exploring the biochemical consequences of irradiation during the silent period between treatment and observation of morphological damage. The survey included determination of total protein nitrogen in the embryos, total DNA in the embryos and rate of incorporation of tritiated thymidine following irradiation. The experimental procedure was as follows: embryos were irradiated at 36-38 hours after onset of incubation with a single dose of 800 roentgens at a dose rate of 35r per minute. Following irradiation, the eggs were returned to the incubator for either 1 1/2 hours or for 3 hours before explantation to the surface of a liquid culture medium to which tritiated thymidine had been added. Embryos were cultured on 1 ml of medium containing 1.25 microcuries of tritiated thymidine. Embryos in explants were again returned to the incubator for a thirty minute labeling period. After this time the embryos were trimmed from their extra-embryonic membranes and pooled according to the developmental stage as determined by the number of somites. The total protein was determined by nephelization and DNA, following cold TCA extract, was determined by the diphenylamine reaction.

These 30 minute explants, studied 2 and 3 1/2 hours after irradiation at 36 to 38 hours after the onset of incubation, showed the following: 1) the total protein nitrogen and total DNA in explants was lower than controls 3 1/2 hours after receiving 800 roentgens of irradiation. The difference, however, was not statistically significant; 2) the synthesis of DNA, as indicated by percent incorporation of tritiated thymidine, was reduced 25 percent by 2 hours and 52 percent by 3 1/2 hours after irradiation; 3) the synthesis of DNA, corrected for the amount of total protein, was depressed by 2 hours after irradiation.

One cannot interpret whether irradiation directly depressed the rate of DNA synthesis or whether irradiation rendered embryos more sensitive to the handling involved in explantation. In either event, irradiation accounts for the differential. The data show that depression in rate of synthesis of DNA

was more pronounced than the decrease in the total of DNA in the embryo. The depression in the rate of synthesis of DNA at 2 and 3 1/2 hours after irradiation is known to be associated with deformities described in the first half of the experiment.

Depression in rate of DNA synthesis could reflect (a) the death of a large proportion of cells, 25 percent in the case of those observed 2 hours after irradiation and 52 percent in those observed 3 1/2 hours after irradiation. This is an unusually high proportion of dead cells in what appear to be viable embryos in explant; (b) prolongation of cell cycles in other than the synthesis period; (c) enlargement of the nucleotide pool as a consequence of irradiation. If irradiation produced degradation of nucleic acids to the extent of doubling the nucleotide pool, one would expect that the incorporation of tritiated thymidine would be reduced by one-half. The present data do not permit distinction between the three possibilities or the extent to which all three possibilities participate to produce the results observed.

Subsequent isotope incorporation experiments are planned to study the relation of DNA synthesis to protein synthesis. Autoradiographic studies are designed to distinguish between a high rate of cell death and specific inhibition of DNA synthesis in otherwise intact viable cells.

This experiment on isotope uptake and protein and DNA synthesis indicates feasibility of studying whole embryos promptly after teratogenic treatment. Refinement of techniques should enable studies on parts of embryos (brain, heart, limb buds) to correlate biochemical changes with subsequent deformity.

B. Thalidomide Derivatives

It is shocking to realize that 14 years after the thalidomide episode we have no sound indication of the mechanism by which thalidomide produced phocomelia. Indirect evidence (Kerberle 1964) suggests that thalidomide, relatively insoluble and easily transported through cell membranes, becomes trapped and concentrated within cells. (Jurand '66 has shown that mitochondria are promptly affected.) Since thalidomide undergoes nonenzymatic hydrolysis and these products may be the teratogens, the following experiments were performed.

Preliminary treatment of explanted chick embryos showed that soluble hydrolytic products of thalidomide produced edema, microcephaly and limb bud defects within 48 hours after exposure.

Four compounds in the first and second steps of hydrolysis were used in comparative tests in equimolar concentrations. Preliminary results indicate that upon direct application to the embryo in vitro the hydrolytic compounds are

teratogenic, that the secondary steps in hydrolysis may be more teratogenic than the primary steps. The grade of teratogenicity also correlates with the number of carboxyl groups on the molecule but such correlation (4 compounds) may be spurious.

This experimental approach to assess teratogenicity and to investigate methods of action of drugs appears to be a fruitful one.

Table 7. Mean score* for chick embryos cultured on equimolar concentrations of hydrolytic products of thalidomide

*The score is a semi-objective one based upon the degree with which the head and brain were affected.

C. Qualitative and Quantitative Separation of Proteins - Microdeterminations in the early chick embryo.

The report last year indicated results of total protein and total DNA and isotope (C^{14} amino acid, H^3 thymidine) uptake by 10 somite embryos during successive periods in culture.

During the past year we have concentrated on protein fractionations preliminary to comparing treated (e.g. X-irradiation) and nontreated embryos. The procedure is not adequately refined but in general is as follows (all procedures are done in the cold room):

1. Trim embryos at the zona pellucida to remove the yolk sac and extra-embryonic membranes.
2. Homogenize in phosphate buffered saline, pH 8.0.
3. Centrifuge 12,000 g. Discard the precipitate containing cell fragments.
4. Half saturate the supernate with ammonium sulfate and centrifuge. Save the precipitate (globulins).
5. Fully saturate the supernate with ammonium sulfate and centrifuge. Save the precipitate (albumins).
6. Resuspend each precipitate with buffered saline and apply to separate sephadex G200 columns.
7. Collect 1 ml effluents (4). Measure amount of protein (Lowry).
8. Apply each fraction to DEAE cellulose column (standardized quantity of protein and carrier volume).
9. Use successive gradients:
 - A. 0.005 M NaH_2PO_4 , $NaHPO_4$ at pH 7.0
to
 - B. 0.2 M NaH_2PO_4 , $NaHPO_4$ at pH 5.7
to
 - C. 0.2 M NaH_2PO_4 , $NaHPO_4$ plus 0.5 M NaCl at pH 5.4
to
 - D. 0.2 M NaH_2PO_4 , $NaHPO_4$ plus 0.8 M NaCl at pH 5.0
to
 - E. 0.2 M $NaHPO_4$, $NaHPO_4$ plus 0.8 M NaCl at pH 4.2
10. Measure proteins in each tube (see graph of tube numbers).
11. Put fractions on sephadex G50 column to desalt and resuspend in diluted tris glycine buffer.

12. Collect effluent. Measure protein (Lowry). Lyophilize. Accumulate fractions for acrylamide gel electrophoresis.
13. Apply 100 µg protein in 0.1 ml to electrophoresis tube. Run, stain and record bands. (See densitometer tracing).

This procedure is a 4 stage fractionation of soluble proteins; (1) ammonium sulfate; (2) sephadex G200; (3) DEAE cellulose and (4) gel electrophoresis. The total number of proteins in usable quantity is large. The procedure is not adequately refined to give the exact number. Column chromatography monitored by electrophoresis provides a powerful tool. This, combined with isotope labels to give specific activities of protein fractions, offers tremendous opportunity to detect effects of irradiation and other treatments on protein synthesis in the early embryo.

Table 8. Effluent from DEAE Cellulose Column, 4.8 mg protein from 96 hour chick embryo
 280 mμ, Flow rate 0.5 ml per minute, Ammonium sulphate fraction
 40-90% saturation.

Table 9. Densitometer tracing of disc gel electrophoretic separation of DEAE fraction from 96 hour chick embryo.