MICROMELIA AND GROWTH RETARDATION AS INDEPENDENT EFFECTS OF SULFANILAMIDE IN CHICK EMBRYOS

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Experimentally induced teratological conditions may be of considerable value for revealing relationships in developmental physiology if one can discover which metabolic processes are disturbed by the treatments that cause the malformation and, if it is possible, to associate these with the abnormal morphology. Should aberrant physiological processes be detected one may explore them in order to determine causal relationships and thus arrive at some picture of the premorphological events which lead to the anomalous development. This type of approach may also reveal which factors are important in normal morphogenesis since, in the absence of other evidence, one must assume that anomalies result from disturbances in the normal developmental processes. Students of experimental teratology have been impressed with the fact that similar syndromes may occur after a variety of treatments. Such observations have been the basis for hypotheses which claim that general or non-specific reactions (i.e., growth retardation or arrested development) are responsible for the appearance of the anomalies. It is only through a study of their derangement of pre-morphological processes that one may actually determine whether different treatments which produce similar conditions do so as the result of non-specific reactions or by

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specific effects which, at some point, have a common or similar action.

It was with these considerations in mind that we undertook a study of the sulfanilamide-induced micromelia which was first reported by Ancel and Lallemand ('42). This chondrodystrophy-like condition (frequently associated with parrot-beak and syndactyly) was produced when solutions of several sulfonamides (including sulfanilamide) were dropped on to the blastoderms of 48-hour chick embryos. The micromelia obtained by this treatment is quite similar, in gross aspects, to that produced by injections of insulin into the yolk-sacs of 4–7-day-old chick embryos (Landauer, '47). It has been found (Landauer, '48) that the micromelic effects of insulin may be obliterated or reversed by simultaneous or subsequent injections of nicotinamide and of α-ketoglutaric acid (the former being considerably more effective in this regard). In addition the insulin produces, in the embryo, an hypoglycemia which may persist for as long as 8 days; the incidence and degree of micromelia is related to the extent and duration of the hypoglycemia and factors which accentuate the low blood sugar also increase the production of micromelia and vice versa (Zwilling, '48, '49). Landauer ('45) demonstrated that when insulin is injected into the yolk-sac of early embryos (2 units administered from 0–64 hours of incubation) the chief effect is the production of rumplessness. Micromelia resulted when older embryos were treated with two units of insulin.

Some of the questions for which we sought answers in this work with sulfanilamide were:

1. Can the sulfanilamide produce its effects after it is injected into the yolk-sac?
2. Will it also produce rumplessness when injected into younger embryos?
3. Does the sulfanilamide alter the blood sugar?
4. Can its micromelia-inducing effects be modified with nicotinamide?
5. Can the micromelia-inducing effects of sulfanilamide be modified by para-aminobenzoic acid?

When our preliminary attempts to answer these questions revealed that sulfanilamide also caused a decrease in size of the embryos we gathered data which is of some significance for the problem of the role of growth retardation in the etiology of terata.

MATERIAL AND METHODS

Eggs from unselected White Leghorn chickens were used for all these experiments. They were incubated on their side in a forced draft incubator. The sulfanilamide (Lederle's) and other substances were injected into the yolk-sac through the blunt end of the egg following the usual virological procedure. Injections were made with syringes equipped with 20 gauge 1-inch long needles. The hole in the shell was sealed with a drop of nail polish. In most instances the concentrations of the substances used were adjusted so that the desired dose was contained in 1/5 cm^3 of solution.

Blood sugar assays were performed by means of the technique described by Zwilling ('48). In order to obtain some quantitative estimate of the growth effects of the various treatments all surviving embryos were removed from their shells at 14 days and weighed. They were then fixed in 10% formol. For data on the micromelic effects the embryos were classified, by inspection, into three categories: (1) normal or slight, (2) moderate and (3) extreme micromelia. After they were fixed the left leg was dissected from each specimen, the muscles stripped from it, and the length of each of the three long bones measured with calipers. In many sulfanilamide-treated embryos the tibiotarsus is bent; in measuring these the part of the bone on each side of the bend was measured and the total length was recorded.

Most of the data are presented along with the controls for the particular experiment. In only a few instances are data from different experiments lumped together. This will account for some of the differences which may be observed after similar treatments. Apparently the susceptibility of eggs from the

same hens varies from time to time. This sort of phenomenon has been demonstrated before (Landauer, '43 has shown that seasonal differences in susceptibility may exist).

Dose effects

Preliminary experiments revealed that the sulfanilamide was effective when it was injected into the yolk-sac of 5-day embryos. Various doses (from .5 to 2 mg) were injected into embryos at this stage and their effects are shown in tables 1 and 2.

TABLE 1

Effect of increasing dose of sulfanilamide on 5-day embryos

DOSE	NO. OF SURVIVORS AT 14 DAYS	% SURVIVAL AT 14 DAYS	% MICROMELIA	% EXTREME MICROMELIA
mg		%	%	%
.5	13	65	0	0
1.0	13	65	46.1	7.6
1.5	21	91	80.9	38.0
1.6	19	63	68.4	15.7
1.8	35	58	85.7	42.8
2.0	29	63	89.6	41.3
Untreated controls	68	94	0	0

Not all of these experiments were performed at the same time, but each group had its own controls and these are all included in the tables. The embryos which received 1.5 mg of sulfanilamide were injected at the same time as those which were treated with .5 and 1.0 mg. The increased survival in this group may represent a sampling variation since it is out of line with the rest. However, it serves to emphasize one of the observations which can be made from these data, namely that the teratogenic effect of the sulfanilamide is independent of its toxic effect. Evidently the latter is not related to dose in the range employed by us while the production of micromelia definitely is. Such a separation of teratogenic action and toxicity has been demonstrated by Ancel for a number of substances and by Landauer for insulin.

In table 2 the data for embryo weight and length of left leg are presented for these different doses. These figures represent all treated embryos, including those with relatively slight effects. Retardation in body size and reduction in leg length were both exaggerated as the dose was increased. With 4 of the 6 doses leg length was affected slightly more than body weight while with 1.6 mg and 2.0 mg the reverse was true.

TABLE 2

Effects of increasing dose of sulfanilamide on embryo weight and leg length [1]

DOSE	AV. WT. (IN GRAMS)	RANGE (IN GRAMS)	% OF CONTROLS	AV. LENGTH LEFT LEG [2] (IN CM)	RANGE (IN CM)	% OF CONTROLS
mg			%			%
.5	8.94	6.64–10.17	95.6	3.56	2.69–3.84	93.4
1.0	7.94	4.59–9.66	84.9	3.14	2.53–3.86	82.6
1.5	7.70	6.40–9.41	82.3	2.87	2.44–3.41	75.5
1.6	6.92	5.52–8.83	74.0	2.91	2.01–3.74	76.5
1.8	7.31	5.19–9.07	78.2	2.89	2.38–3.63	76.0
2.0	5.75	3.71–7.42	61.4	2.55	1.68–3.55	67.1
Untreated controls	9.35	6.88–11.79	...	3.80	2.94–4.19	

[1] These data are for all embryos, including non-reactors.

[2] Combined lengths of femur, tibiotarsus and tarsometatarsus.

Influence of stage treated

In the following experiments a uniform dose of sulfanilamide (1.6 mg) was injected into embryos of different ages. The younger embryos were treated in an attempt to see whether injected sulfanilamide resulted in an increase in rumplessness. Diagnosis for rumplessness was made in the survivors of the 4th day of development in the case of embryos which had been injected at 30 and 48 hours after incubation had begun. A total of 111 embryos were injected at 30 hours and 83 at 48 hours. There were 45 controls. Among the 92 survivors of the 4th day in the 30-hour group 7 (7.6%) were rumpless. Three (4.0%) of the 75 survivors of the 4th day in the 48-hour group were rumpless. None of the untreated controls had this abnormality. In 113 untreated controls from

other experiments (examined at the 14th day) two were rumpless (1.7%). The 7.6% incidence after treatment of 30-hour embryos is obviously significant. Moseley ('47) found that 5.4% of her controls (56 embryos) were rumpless. The 1.7% which we encountered in the controls corresponds better with the incidence which Landauer and Baumann ('43) reported. When embryos treated at 48 hours are compared with our controls it is seen that the sulfanilamide does enhance the occurrence of rumplessness somewhat.

TABLE 3

Micromelic and survival effects of injection of 1.6 mg of sulfanilamide on embryos of different ages

AGE OF EMBRYOS WHEN TREATED	NO. OF SURVIVORS AT 14 DAYS	% SURVIVAL AT 14 DAYS	% MICROMELIA	% EXTREME MICROMELIA
		%	%	%
30 hours	48	43	77.0	52.0
48 hours	35	47	94.3	60.0
5 days	19	63	68.4	15.7
7 days	13	92	0.0	0.0
9 days	12	92	0.0	0.0
Untreated controls	65	91	0.0	0.0

From the data presented in tables 3 and 4 it can be seen that the total incidence of micromelia, and especially that of the extreme type, was increased when the younger stages were treated. The mortality was somewhat greater at these stages than later on. It is interesting to note that there was no micromelia when 7- and 9-day embryos were injected with 1.6 mg of sulfanilamide. This is more evident in table 4, where it can be seen that the leg length of these embryos was relatively unaffected. Note, though, that the sulfanilamide still exerted an effect on body size in these older embryos; when injected at 7 days the embryos attained only 86.6% of the control weight while those injected at 9 days weighed 90.4% as much as the controls. In these embryos the legs were relatively larger than those of the controls. On the other hand, in embryos in-

jected at 30 and 48 hours, the legs were affected relatively more than the body size. This difference also holds between these embryos and the ones treated at 5 days and reflects the greater preponderance of extreme micromelia.

Blood sugar effects

Assays for blood sugar were performed on embryos which had been injected with 1.6 mg of sulfanilamide at 5 days. Blood was obtained from 9 6-day, 10 8-day, 5 10-day, 5 12-day

TABLE 4

Weight and leg-length effects of injection of 1.6 mg sulfanilamide on embryos of different ages [1]

AGE OF EMBRYO WHEN TREATED	AV. WT. (IN GRAMS)	RANGE (IN GRAMS)	% OF CONTROLS	AV. LENGTH LEFT LEG (IN CM)	RANGE (IN CM)	% OF CONTROLS
			%			%
30 hours	6.88	4.25–10.49	71.7	2.63	1.00–4.23	67.9
48 hours	6.57	3.96–8.77	68.5	2.49	1.89–3.17	64.3
5 days	6.92	5.52–8.83	72.1	2.91	2.01–3.74	75.2
7 days	8.31	6.88–9.85	86.6	3.77	3.36–4.15	97.4
9 days	8.67	5.77–9.96	90.4	3.84	2.99–4.28	99.2
Untreated controls	9.75	6.67–11.79	...	3.89	3.09–4.22	...

[1] These data are for all embryos, including non-reactors.

and 5 14-day embryos. We also included, at 6 and 8 days, some embryos (6 and 5) which had received 2.5 mg of para-aminobenzoic acid in addition to the sulfa. None of the blood sugar values from these determinations were abnormal. Most of the embryos survived to 14 days and could be diagnosed for micromelic effects of the sulfa. Eight of them were extreme micromelics and several were classed as near-extreme. This evidence was considered sufficient to indicate that sulfanilamide does not affect the blood sugar of the embryos and further assays were not attempted.

Effects of para-aminobenzoic acid

One of the current hypotheses about the inhibition of bacterial growth by sulfanilamide is that it is an antagonist of para-aminobenzoic acid. It has been demonstrated that addition of excess PABA may, in most cases, counter the action of the sulfa drug. We were curious to know whether the growth and micromelic effects of the sulfanilamide could be likewise altered by PABA. The PABA was converted to its sodium salt, which is more soluble than the acid, by addition of NaOH

TABLE 5

Effect of PABA on sulfa-induced micromelia

TREATMENT	NO. OF SURVIVORS AT 14 DAYS	% SURVIVAL AT 14 DAYS	% MICROMELIA	% EXTREME MICROMELIA
		%	%	%
1.8 mg sulfa	27	60	62.9	33.3
1.8 mg sulfa + 7.5 mg PABA	8	21	62.4	37.5
7.5 mg PABA	22	34	0	0
7.5 mg PABA + 5 mg nicotinamide	10	14.7	0	0
Untreated controls	24	96	0	0
5 mg PABA at 48 hours	21	42	0	0
Untreated controls	44	83	0	0

to the solution. This enabled us to increase the dose without increasing the volume excessively. After preliminary trials a dose of 7.5 mg PABA in 1/5 cm^3 was employed.

As outlined in tables 5 and 6 embryos of 5 days were subjected to the following treatments:

(1) Sulfanilamide alone, (2) sulfanilamide + PABA, (3) PABA alone, (4) PABA + nicotinamide (which, as we shall demonstrate, has an effect on the micromelia-inducing action of sulfanilamide). Group 5 consisted of untreated controls. At a later time a group of 48-hour embryos was injected with 5 mg of PABA. These had their own controls.

Para-aminobenzoic acid did not alleviate any of the effects of sulfanilamide in the chick embryos. In fact it accentuated them. This is evident from the increase in mortality and the exaggeration of growth retardation. The incidence of micromelia was not augmented, but the average leg length was decreased, probably a reflection of the decrease in body size.

TABLE 6

Effect of PABA on weight and leg-length of sulfanilamide-treated embryos [1]

TREATMENT	AV. WT. (IN GRAMS)	RANGE (IN GRAMS)	% OF CONTROLS	AV. LENGTH LEFT LEG (IN CM)	RANGE (IN CM)	% OF CONTROLS
			%			%
1.8 mg sulfa	7.66	6.04–10.90	83.8	3.05	2.13–4.01	81.3
1.8 mg sulfa + 7.5 mg PABA	5.88	4.41–6.88	64.3	2.55	2.25–3.35	68.0
7.5 mg PABA	7.12 [2]	4.00–10.32	77.8	3.18 [3]	2.40–3.60	84.8
7.5 mg PABA + 5 mg nicotinamide	7.28	4.40–11.09	79.6	...	....	...
Untreated controls	9.14	6.75–11.07	...	3.75	2.94–4.07	...
5 mg PABA at 48 hours	8.42	6.18–10.25	93.3	...	....	...
Untreated controls at 48 hours	9.02	7.43–10.31	...	..	....	...

[1] These data are for all embryos, including non-reactors.
[2] N = 22.
[3] N = 7.

Quite unexpectedly is was found that PABA alone caused a growth retardation but was not effective in producing micromelia. In the dose employed in the experiment with 5-day embryos the PABA reduced body weight quite as effectively as did sulfanilamide. Although the legs were small (but relatively less reduced than the body) they were normally proportioned. The simultaneous administration of nicotinamide with

PABA did not alter its weight-reducing effects. It can be seen that PABA alone was very toxic and that in combination with either sulfanilamide or nicotinamide its lethal effects were increased considerably.

In order to find whether the absence of micromelia following injections with PABA might be dependent on the time when it was administered another group of eggs was injected at 48 hours. The amount of PABA was decreased to 5 mg in an attempt to increase survival. Again we found that there was no micromelia even though the mortality was high and there was a decrease in weight of the embryos, though not quite so marked as when older embryos were treated.

Effects of nicotinamide

In this next set of experiments we attempted to discover whether nicotinamide had the same anti-micromelic action with sulfanilamide as it does with insulin. To this end nicotinamide was injected into 5-day embryos immediately after they had been treated with sulfanilamide. Various doses were tried and finally a dose of 5 mg was selected. Higher doses were exceedingly toxic in combination with the sulfa-drug. From the few survivors obtained with the greater doses it could be seen that the results were essentially the same as those obtained with 5 mg.

Ancel ('45) has advanced the idea that the sulfanilamide was exerting its effect on the pre-cartilaginous mesenchyme during the two days after its application (i.e., from day 2 to day 4). According to him, therefore, the micromelia is an expression of the drug's action on the undifferentiated tissue. In view of the fact that embryos may become micromelic when treated as late as 5 days this did not seem a likely interpretation. When it was found that nicotinamide did eliminate the sulfa-induced micromelia we planned an experiment in which sulfanilamide was injected at one time and nicotinamide several days later.

Tables 7 and 8 contain the data for experiments with nicotinamide. The results are completely unequivocal. No embryos

which received nicotinamide in addition to sulfanilamide developed micromelia. This was equally true in our preliminary work with different doses. However, it is quite interesting to note that the nicotinamide acted selectively in preventing or alleviating the micromelia but did not affect the growth retarding action of sulfanilamide appreciably when both substances were injected into 5-day embryos. When the nicotinamide was injected along with sulfanilamide at 48 hours or at

TABLE 7

The effect of nicotinamide on sulfa-induced micromelia

TREATMENT	NO. OF SURVIVORS AT 14 DAYS	% SURVIVAL AT 14 DAYS	% MICROMELIA	% EXTREME MICROMELIA
		%	%	%
Expt. 1				
1.8 mg sulfa	21	70	85.7	42.8
1.8 mg sulfa + 5 mg nicotinamide	22	53	0.0	0.0
Untreated controls	11	100	0.0	0.0
Expt. 2				
1.6 mg sulfa at 48 hours	15	50	93.3	60.0
1.6 mg sulfa + 5 mg nicotinamide at 48 hours	10	24	0.0	0.0
1.6 mg sulfa at 48 hours + 5 mg nicotinamide at 5 days	9	25	0.0	0.0
Untreated controls	15	93	0.0	0.0
Expt. 3				
1.8 mg sulfa at 5 days	30	70	36.6	16.6
1.8 mg sulfa at 5 days + 5 mg nicotinamide at 6 days	24	56	0.0	0.0
1.8 mg sulfa at 5 days + 5 mg nicotinamide at 7 days	26	60	0.0	0.0
Untreated controls	10	83	0.0	0 0

5 days, after the sulfanilamide had been injected at 48 hours, there was some increase in body size. However, the leg length was increased proportionately more. When the sulfanilamide was injected at 5 days and the nicotinamide at 6 and 7 days

TABLE 8

The effect of nicotinamide on weight and leg length of sulfanilamide-treated embryos [1]

TREATMENT	AV. WT. (IN GRAMS)	RANGE (IN GRAMS)	% OF CONTROLS	AV. LENGTH LEFT LEG (IN CM)	RANGE (IN CM)	% OF CONTROLS
			%			%
Expt. 1						
1.8 mg sulfa	7.14	5.19–9.00	71.4	2.88	2.29–3.63	73.2
1.8 mg sulfa + 5 mg nicotinamide	7.69	6.50–9.80	76.9	3.61	3.25–3.88	91.8
Untreated controls	10.00	8.68–11.51	...	3.93	3.78–4.19	...
Expt. 2						
1.6 mg sulfa at 48 hours	6.66	3.96–8.77	63.8	2.68	1.95–3.17	67.8
1.6 mg sulfa + 5 mg nicotinamide at 48 hours	7.85	6.42–8.98	75.2	3.61	3.18–3.90	91.4
1.6 mg sulfa at 48 hours + 5 mg nicotinamide at 5 days	8.26	6.35–10.31	79.2	3.74	3.24–4.11	94.6
Untreated controls	10.43	7.36–11.78	...	3.95	3.10–4.25	...
Expt. 3						
1.8 mg sulfa at 5 days	8.28	5.46–9.93	77.4	...	....	...
1.8 mg sulfa at 5 days + 5 mg nicotinamide at 6 days	8.77	6.20–10.60	82.0	...	....	...
1.8 mg sulfa at 5 days + 5 mg nicotinamide at 7 days	8.59	6.55–10.34	80.3	...	....	...
Untreated controls	10.69	9.06–12.17	...	...	..	...

[1] These data are for all embryos, including non-reactors.

there was no micromelia (the leg length measurements for these embryos were not recorded) and the weight of the embryos was not improved to any great extent.

Effect of nicotinamide on the beak

When embryos were treated with sulfanilamide alone or together with PABA, many of the more extreme micromelics also had parrot-beaks. When nicotinamide was administered either immediately following, or some time after, the injection of sulfanilamide the parrot-peak condition was no longer found. Instead we encountered a considerable incidence of shortening of the upper beak. This was true even when the nicotinamide was injected on days 6 and 7 and sulfanilamide on day 5. From a total of 97 embryos treated with both sulfanilamide and nicotinamide 48 (49.4%) had shortened upper beaks. This condition was seen in only two of 140 untreated control embryos (1.4%) utilized in these experiments and in 5 of 424 embryos (1.17%) treated with only sulfanilamide or PABA or both. The degree of shortening of the upper beak in controls and in sulfa- and PABA-treated embryos was, for the most part, considerably less marked than in embryos treated with nicotinamide.

Landauer ('48) found, when insulin and nicotinamide were administered either simultaneously or at intervals of 3 hours at between 24 and 30 hours of incubation, that the incidence of rumplessness was considerably reduced but that shortening of the upper beak occurred in 5–12% of the embryos. This treatment did not produce short upper beaks in 96- or 120-hour embryos. In the present material the incidence of short upper beak was high even when the nicotinamide was injected at 7 days (34%) (sulfanilamide at 5 days). Landauer has also found (unpublished) that nicotinamide eliminated the parrot-beak condition which followed injections of eserine sulphate, and that shortening of the upper beak was frequently encountered. We did not inject embryos with nicotinamide alone

since Landauer had performed these control experiments and we had repeated them in connection with other work. Nicotinamide alone did not produce any abnormalities.[3]

Structural analogs of nicotinamide

Ackermann and Taylor ('48) have reported the results of injections of 3-acetylpyridine into chick embryos. They measured the toxic effects of sub-lethal doses (400–550 γ) by determining the fraction of treated embryos which were dead after 24 hours. In addition they stated that "Some of the symptoms associated with the toxicity were undersized deformed legs, and a general edematous-like condition . . ." These effects were reversed by addition of proper amounts of nicotinamide. They did not state whether their embryos were examined at later stages. Since the "undersized deformed legs" sounded like micromelia we were, in light of our results with nicotinamide, quite interested in studying the effect of 3-acetylpyridine on blood sugar, etc.

Our first step was to inject some 3-acetylpyridine to see whether micromelia was actually produced. Doses ranging from 400 γ to 800 γ were injected into 4- and 5-day embryos, which were then examined at 14 days. In no case was micromelia found. The embryos were reduced in size and many were edematous. However, the effect on the limbs was quite unlike that seen after injection with other substances. The wings were articulated in such a manner that they lay extended along the back of the embryo. The hind-limbs were normal in length for embryos of this size but were thinner than normal. Most of the other bones (ribs, etc.) were also thinner than normal. We have not made a detailed study of these effects.

The effect of 3-acetylpyridine on the weight of the embryos cannot be measured accurately because of the high incidence of edema; one cannot drain off the fluid. Some rough idea may be obtained from the following: the average weight at 14 days

[3] Since this was written Landauer (unpublished), in another series of experiments, found that 5 mg of nicotinamide alone produced short upper beaks in 7% of embryos treated at 24 hours and in .09% of embryos injected at 96 hours.

of 75 embryos treated with from 400 γ to 800 γ (27 with 400 γ, 35 with 500 γ, 6 with 600 γ and 7 with 800 γ) and weighed with edematous fluid was 7.08 gm. That for 21 untreated controls (none edematous) was 8.75 gm. The weight reduction was about the same whether the injection was made at 4 days or at 5 days. The averages for the weights for doses 400, 500, and 600 γ were about the same (7.03, 7.20 and 7.05 gm, respectively) while that for 800 γ was 6.77 gm.

Curiously enough 3-acetylpyridine caused an increase in the incidence of shortened upper beaks. Sixteen per cent of all embryos treated with this substance had such a condition. Only one (4.7%) of the 21 control embryos had a short upper beak.

One other antagonist of nicotinic acid which we tried was pyridine-3-sulfonic acid (which was used in its acid form). Doses of from 200 γ to 80 mg were injected into small groups of embryos. Aside from an evident toxicity with doses in excess of 30 mg there was no appreciable effect on the embryos after they were treated with pyridine-3-sulfonic acid.

Size of long bones in micromelic embryos

In order to compare the shortening of the long bones of the hind limbs in our experimental material with the data which Landauer ('39) has presented for Creeper embryos, 20 extreme and 20 moderate cases of sulfanilamide-induced micromelics as well as 20 controls were selected at random from our material. Two types of calculations were made: from the averages of their lengths the proportion of the individual bones to the total length of all three elements was obtained and the reduction of each bone relative to the total reduction was calculated

$$\left(\frac{\text{Control bone} - \text{treated bone} \times 100}{\text{Combined length of control bones} - \text{combined length of treated bones}}\right).$$

From table 9 it may be seen that the relative proportions of the bones was not altered very greatly in moderate micromelics. In the extremes the femur was relatively longer and

the other two bones shorter than in the controls. The latter is essentially what Landauer found for homozygous Creeper embryos. However, our moderates showed less change in proportion than did the heterozygous Creepers. Our controls, too, differed from Landauer's normal embryos from the Creeper matings; the femur is relatively larger and the other two

TABLE 9 [1]

Proportion of total length for each leg bone $\left(\frac{\text{length bone} \times 100}{\text{combined length}}\right)$

	TOTAL LENGTH	FEMUR	TIBIOTARSUS	TARSOMETATARSUS
	cm			
Controls	3.78	31.14	39.85	28.88
Moderate micromelics	2.85	31.73	37.20	28.99
Extreme micromelics	2.33	36.14	36.79	26.86

[1] All embryos 14 days old.

TABLE 10 [1]

Reduction in length of leg bones relative to total reduction (see text)

	TOTAL REDUCTION	FEMUR	TIBIOTARSUS	TARSOMETATARSUS
	cm			
Moderate micromelia	.93	23.6	48.4	27.9
Extreme micromelia	1.46	23.4	44.8	31.7

[1] All embryos 14 days old.

bones somewhat smaller in our embryos. The tibiotarsus and tarsometatarsus in the extreme micromelics (table 10) were shortened relatively more than the femur, with the tibiotarsus showing the greatest shortening. The same was seen in the moderates, although in this case the tibiotarsus was even more shortened relative to the others and the tarsometatarsus relatively less than in the extremes. Again this is essentially the

same as in Creeper embryos of the same age. The extreme micromelics show the same proportionate reduction as do the homozygous Creepers. In our moderates the femur is relatively more shortened and the tarsometatarsus relatively less shortened than they were in the heterozygous Creepers, while the tibiotarsi in the two were similar in this respect.

The same calculations were performed for 18 embryos treated with sulfanilamide and nicotinamide at 5 days. The comparison was made with 11 controls incubated at the same time. The total average reduction for all three bones of the limb in the treated embryos was .29 cm. The proportions were very close to those for the controls: (in the following figures control proportions are given first) femur, 30.7–31.6; tibiotarsus, 39.2–38.6; tarsometatarsus, 29.8–29.5.

These data demonstrate that the growth changes in the limbs of sulfanilamide-induced micromelia are very similar to those observed in Creeper embryos. The tibiotarsus is most reduced and the femur shows the least reduction.

DISCUSSION

The interpretation of developmental studies of the type described in this paper depends, to a great extent, on information obtained from experiments performed on adult organisms. There is no complete analysis of the function of such substances as nicotinamide in the embryo. Levy and Young ('48) have demonstrated that all of the nicotinic acid (or amide) in chick embryos is in the form of the diphosphopyridine nucleotide. It has been known for some time that DPN (or coenzyme I) is of extreme importance in both carbohydrate and amino acid metabolism (Ball, '39; Krehl, '49). Lennerstrand ('41) had shown, in vitro, a relationship between the degeneration and regeneration of DPN and glucose. Under proper conditions (presence of apoenzyme) phosphated glucose enhances the synthesis of the DPN, while, conversely, its absence inhibits it. These, then, are the salient facts upon which we must base an interpretation of our results with nicotinamide. (Wald ['49] has shown another function of DPN, in the syn-

thesis of A vitamins from retinenes, but too little is known of any general application to steroid synthesis to warrant considering it here.)

Sulfanilamide's inhibition of bacterial growth has been interpreted to be the result of a competition for PABA. Since PABA does not alleviate any of the effects of sulfanilamide in chick embryos it is evident that the action of the sulfa is not a result of PABA antagonism. In fact, PABA itself acts as an inhibitor of growth in chick embryos. It has been known for some time that certain ill effects of sulfanilamide therapy may be alleviated by treating patients with nicotinamide. This points to a dual effect of sulfanilamide, since its inhibition of bacterial growth does not seem to be appreciably altered when the nicotinamide is administered. Our results with chick embryos also indicate a dual effect of sulfanilamide: one on general embryonic growth and one on the development of the long bones of the limbs. Only the latter is affected by nicotinamide therapy. It seems possible that the growth inhibition of both PABA and sulfanilamide may be the result of these similar molecules interfering with the same substance or process, while the micromelic effects are due to some specific action of the sulfanilamide.

Several possible explanations of the sulfanilamide's micromelia-inducing effects and their antagonism by nicotinamide present themselves: the sulfanilamide may inhibit DPN synthesis directly, it may interfere with amino acid metabolism or it may interfere with carbohydrate metabolism. In any of these possibilities the addition of nicotinamide could be helpful in overcoming the effects. Apparently DPN-mediated metabolism is more important in chondrogenesis of long bones than in other growth or differentiation processes since the nicotinamide did not alleviate the general growth inhibition.

Sulfanilamide, unlike insulin, does not affect the embryonic blood sugar although it does cause a similar morphological effect. Aside from the production of micromelia the only physiological similarity between the two substances is the fact that their effects are reversed by nicotinamide. Recently

Landauer (unpublished) has found that the micromelia which is induced by injections of eserine sulfate is also eliminated by nicotinamide. It is not clear, at this point, whether the insulin-induced hypoglycemia is a direct cause of the micromelia. This seems unlikely in view of the action of nicotinamide. It is more likely that the decreased hexose content of the blood (and, one assumes, the tissues) causes an increased requirement for DPN either through a more rapid destruction or an inhibition of its synthesis under these conditions (see Lennerstrand's work above). There is another possibility: the insulin may alter the amino acid metabolism directly. Harris and Harris ('47) have shown that amino acids in the blood of humans may be altered by insulin. At the present stage of these investigations one can but hypothecate that these substances, whether by direct antagonism or by indirect action on carbohydrate or amino acid metabolism, achieve a common result, namely that of interfering with a process which is either dependent on a nicotinamide containing compound or for which such a compound may provide an alternate source of energy.

We had hoped that a direct antagonism of nicotinamide by some of its antimetabolites might be very revealing for our problem. The fact that 3-acetylpyridine and pyridine-3-sulfonic acid did not produce micromelia does not necessarily eliminate this approach. It is well known that antimetabolites do not have the same effects in all animals or under different dietary conditions. In a system such as one finds in a normal chick embryo, where all nutrients are present in a relatively closed system, the action of antimetabolites may conceivably be altered.

For years teratologists have been concerned with the role of developmental retardation as a cause of malformations. It has become customary to consider the time when a substance acts to be more important than any specificity in its action. In recent years evidence has been brought forth (largely by Ancel and his group) which indicates that the specific action of the teratological agent may be equally effective in determining

the nature of an abnormality and that one must consider both factors. The data which we have presented in this paper clearly indicates that the micromelic effects of sulfanilamide are separate from its growth retarding effects. Two facts support this strongly: nicotinamide selectively reverses only the effects on the limbs; and PABA produces a growth inhibition without any accompanying micromelia. It is unlikely that differences in solubility or in transport of sulfanilamide and PABA to the embryo may be responsible for any variations in their effects since PABA had no micromelia-inducing effect when injected at either 48 hours or 5 days. This point is strengthened by our findings that sulfanilamide is effective in producing micromelia over a wide period of time: from 30 hours to 5 days. We can conclude, in the case of sulfanilamide-induced micromelia, that the growth retardation is not involved in the production of the abnormality but that some specific action of the drug is the causal factor.

It is of some interest to note that insulin and sulfanilamide differ both in regard to the time of treatment which produces a maximum incidence of micromelia and the span of time over which they are effective in producing the condition. With insulin the greatest incidence of micromelia was encountered at 120 hours; with sulfanilamide it was 48 hours. After injections of two units of insulin micromelia was produced from 96 hours to 168 hours. (There is some evidence [unpublished] that 5 units of insulin, administered as early as 30 hours, may produce some micromelia; this is associated with an hypoglycemia which persisted as late as the 10-day stage after such treatment.) With sulfanilamide this condition did not appear when embryos older than 5 days were treated.

Ancel interpreted his results with sulfanilamide to indicate that this substance acts on pre-cartilage tissue prior to the elaboration of the limb. The abnormal chondrogenesis, etc., was, according to him, the expression of the earlier influences. This seems unlikely for several reasons. Sulfanilamide may produce micromelia when it is injected into 5-day embryos and the effects of prior injections may be eliminated as late as 7

days, when chondrogenesis has been under way for some time. Insulin may induce micromelia even when it is injected at 7 days (Landauer, '47). Ancel dropped a solution containing 1.25 mg of sulfanilamide on to the blastoderm of 48-hour embryos. Probably most of the solution rolled off of the blastoderm, dissolved in the albumen and possibly was distributed to other parts of the embryo. According to V. Groupé and A. P. Richardson (unpublished data) when sulfadiazine was injected into the yolk-sac of 7-day embryos it was quickly distributed so that within 24 hours it was found in the allantoic fluid, yolk, chorioallantoic membrane, amniotic fluid, embryonic tissue and yolk sac (in order of decreasing concentration). Over a period of 11 days the amount in each region varied, but the sulfadiazine was present in all tissues and fluids assayed. It is possible that sulfanilamide may be distributed in a similar manner. Quite likely the sulfanilamide in Ancel's experiments was available to the embryo over a considerable time and exerted its influence for a period which included the stage when cartilage formation had begun.

SUMMARY

1. Sulfanilamide may produce micromelia, parrot-beak and growth retardation in chick embryos when it is injected into the yolk-sac. The teratogenic effects of sulfanilamide increased as the dose was increased, but the toxic action did not increase accordingly.

2. Micromelia resulted after injections of sulfanilamide at 30 hours and at 5 days. Rumplessness occurred in 7.6% of embryos treated at 30 hours, and 4.0% of those treated at 48 hours. Both the incidence of extreme micromelia and the degree of growth inhibition were greater when earlier stages (48 hours) were treated.

3. Sulfanilamide did not alter the blood sugar when it was injected into 5-day chick embryos.

4. Para-aminobenzoic acid did not interfere with the sulfanilamide effects; it exaggerated the growth inhibition. When PABA was administered by itself, growth of the embryo was retarded.

5. When nicotinamide was injected along with or from 2–3 days after sulfanilamide, the micromelic effects of sulfanilamide were completely eliminated. However, in most instances, the growth retardation was not appreciably affected by this treatment.

6. When nicotinamide was injected in conjunction with sulfanilamide regardless of the interval between injections, cases of parrot-beak were eliminated but there was an increased incidence of shortening of the upper beak; 49.4% of all embryos treated with both substances had this anomaly.

7. Two structural analogs of nicotinamide, 3-acetylpyridine and pyridine-3-sulfonic acid, did not produce micromelia. The former caused a general growth retardation and thinning of the bones.

8. The proportional reduction of the bones of the leg in sulfanilamide-induced micromelic embryos was similar to that of Creeper embryos of the same age.

9. The possible mechanisms responsible for the micromelic condition are discussed. It is emphasized that this study clearly eliminates growth retardation as a causal factor in the production of sulfanilamide-induced micromelia; in this instance it results from some specific effect of the drug.

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