THE SURVIVAL OF AMBLYSTOMA EMBRYOS WHEN TREATED WITH SODIUM SULFADIAZINE AND QUININE SULPHATE[1]

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TWO FIGURES

INTRODUCTION

In a previous communication (Detwiler and Robinson, '45) we emphasized the value of sodium sulfadiazine as a bacteriostatic agent in surgical procedures upon the central nervous system of amphibian embryos. Without sterile precautions we excised the right brachial region of the spinal cord from embryos subsequent to the closure of the neural folds (stages 21–25). The wounds were not covered. The operations were done in 0.4% salt solution and the embryos were transferred later to spring water. Only 3 of 280 operated embryos survived. This discouraging yield prompted us to study the possibility of employing sulfa drugs. After preliminary tests, it was found that embryos would undergo normal development and growth when kept in a 1% solution of sodium sulfadiazine (Sharp and Dohme) made up in spring water.

The identical operations upon similar stages were then repeated. Following the operation (in 0.4 NaCl solution) the embryos were divided into 2 equal groups. The specimens of 1 group were placed in individual Syracuse dishes contain-

[1] This communication is introductory to several papers which will deal with the effects of various drugs on the growth and development of Amblystoma embryos.

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ing a 1% solution of the drug; those of the other group were placed in individual dishes containing spring water. The intervals between the time of operation and transfer to either sodium sulfadiazine or spring water ranged from 2 to 12 hours. In order to eliminate any possible modifying factor due to egg selection, embryos from individual clutches were distributed equally between spring water and the sulfa drug solution following the operation. Of 95 embryos kept in spring water, only 1 survived the fourth day, whereas of those treated with sodium sulfadiazine, only 5 out of 95 succumbed. Embryos transferred from the solution of the drug to spring water 2 to 3 days postoperative, survived as well as did those maintained for longer periods in the sulfadiazine solution.[3]

Observations on the healing of the wounds showed that, although sulfa treatment resulted in a 95% survival, it did delay healing by 1 to 2 days. Embryos in spring water which showed complete superficial wound healing, underwent regional or complete disintegration. The sulfa treated embryos when once healed continued to survive. The results of these experiments which were done during the 1945 operating season were gratifying far beyond expectations. It should be pointed out that in these experiments, all of the eggs came from the same pond. Inasmuch as the experiments were carried out at the close of the operating season, opportunity was not afforded to study the effects of varying concentrations of the drug nor minimal lengths of treatment necessary to insure beneficial effects. Furthermore, there was no opportunity to study the reactions of young embryos (pregastrula, gastrula and plate stages) to the drug, nor the possibly different responses of embryos shipped from different geographical localities.

Experience over many years has shown that the survival of embryos may vary with different clutches of eggs. Some are presumably more hardy than others. The same type of operation, under apparently identical technical procedures, may (with one egg mass) result in low mortality; in another

[3] For complete data see Detwiler and Robinson, op cit., table 1.

it may be high. Evidence from long term experience indicates too that viability may vary with eggs from the same or different localities and from year to year. Careful handling is also an important factor, both in transportation and in the laboratory.

With subsequent use of the drug (1946 operating season) over a range of Harrison's stages from 15 to 25, with considerable variation in the type of operation, and employing embryos from different localities, our survivals have not been so consistently high.

OBSERVATIONS UPON SURVIVAL OF OPERATED EMBRYOS

One set of observations on survival following sulfa treatment was made upon eggs shipped from Durham, North Carolina and Nashville, Tennessee. The operations upon embryos from these sources were done in late February 1946. The type of operation and the stages employed varied considerably. The percentage of survival of sulfa treated embryos as compared with those reared in spring water following a variety of experimental procedures, is given in table 1. A survey of this table will show that in some groups sulfa treatment was followed by a much greater percentage of survivals than untreated embryos from the same egg clutch regardless of the stage when the operation was performed (e.g. groups 3, 4, 8, 9, 10, 11, 13). In other groups the survivals following sulfa treatment was no higher or even less than untreated embryos (groups 2, 6, 7, 12). Since in these groups, the survival of embryos in spring water ranged from 40–50%, which is considered very satisfactory in view of the type of operation, one is inclined to the view that the higher death rate following sulfa treatment in these groups is an index of intolerance to the drug. Further evidence supporting this view is suggested from the results obtained upon sulfa treated normal embryos (vide infra). In general it may be said that embryos, which were operated upon just after closure of the medullary folds and which were subsequently sulfa treated, yielded a higher percentage of survivals than younger em-

TABLE 1

Showing survival of sulfa treated embryos as compared with those nontreated following various operations as indicated in column 2 and explained below.

	TYPE OF EXPERIMENT	STAGE	NUMBER OF OPERATIONS	SOURCE OF EGGS	HOURS AFTER OPERATION WHEN TRANSFERRED TO		NUMBER	DAYS IN SODIUM SULFADIAZINE	SURVIVAL AT END OF 9 DAYS	PER CENT SURVIVAL
					S.D.	S.W.				
1	FBEB	21	10	Durham	2		5	5	5	100
						2	5	—	0	0
2	NCER	20	20	Durham	1½		10	9	4	40
						1½	10	—	4	40
3	FBEB	21	30	Nashville	1½		15	9	12	80
						1½	15	—	1	6.6
4	MPER	15	30	Nashville	2 +		15	3	14	93
						2 +	15	—	7	46
5	NCER	19	53	Durham	3		25	3	20	80
						3	28	—	20	71
6	MPER	15–16	50	Nashville	2½		25	2	4	16
						2½	25	—	10	40
7	MPEB	15–18	26		3		15	2	6	40
						3	11	—	7	46
8	FBEB	21–22	50	Nashville	5		30	4	28	90
						5	20	—	5	25
9	CMR	20–21	25	Nashville	4		15	3	8	53
						2	10	—	1	10
10	MPER	15–16	50	Durham	5		36	5	25	69
						3½	14	—	2	14
11	MPEB	15	50	Durham	5		36	4	31	86
						4½	14	—	4	29
12	NCEB	15–16	60	Nashville	6		40	4	18	45
						5	20	—	10	50
13	FBEB	21	60	Nashville	4		40	4	18	45
						3	20	—	0	0

FBEB = Bilateral excision of forebrain.
NCER = Unilateral excision of trunk neural crest.
NCEB = Bilateral excision of trunk neural crest.
MPER = Unilateral excision of trunk medullary plate.
MPEB = Bilateral excision of trunk medullary plate.
CMR = Spinal cord substituted for medulla (unilateral).
SW = Spring water.
SD = Sodium sulfadiazine (0.5–1.0%).

bryos similarly treated. However this was not exclusively so, for several groups which were operated upon when in stage 15–16 and followed by sulfa treatment, also yielded good results (groups 4, 10, 11). These observations show that many sulfa treated embryos in the medullary plate stage are not harmed by concentrations of the drug as high as 1%, but are actually benefited by the sulfa treatment following the operation. In those embryos of similar stage where the mortality rate was high (groups 6, 12) one can only infer that the embryos were more susceptible to the toxicity of the drug in the dilutions used. Such susceptibility and intolerance with some embryos as compared with others, recalls similar situations encountered in clinical practice, where in one person may tolerate sulfa treatment much more readily than others.

A factor of importance when dealing with early stages pertains to the effects of delayed healing occasioned by the drug. In group 2, the trunk neural crest was excised on one side from embryos with high but unfused folds. Sulfa treatment was seen to definitely hinder the normal cell movements which eventuate in a closed tube. In consequence, spina bifida resulted in many embryos with subsequent death. These events were not encountered with those embryos kept in spring water. The high mortality can thus be associated with abnormality occasioned by delayed development rather than to any general toxic effect. When this same operation or a bilateral operation was done on younger stages, the yield was much better (groups 5, 12).

Embryos following bilateral excision of the trunk medullary plate (group 7) exhibited large persistent anal yolk masses which, in many cases, failed to become properly incorporated in the embryo. This condition was due partly to the operation itself, but the number which failed in this respect was much higher in the sulfa treated embryos than among those reared in spring water. Complete failure was followed by further abnormal development and eventual death. Here again it is seen that the survival may be lower than expected because of abnormalities resulting from delayed

development or inhibition of normal movements caused by the drug.

It will be seen from table 1 that in the operations involving the excision of the forebrain, the percentage of survival was high in all sulfa treated embryos as compared with those reared in spring water (groups, 1, 3, 8, 13). This operation merely involves closing over the wound, which is usually fairly rapid. It does not involve complicated processes such

TABLE 2

A summary of the data in table 1 showing survival of sulfa-treated embryos as compared with non-treated embryos.

	SULFA-TREATED EMBRYOS (0.5 TO 1.0% SODIUM SULFADIAZINE)		NON-TREATED EMBRYOS (SPRING WATER)	
Group	Number	Survived	Number	Survived
1	5	5	5	0
2	10	4	10	4
3	15	12	15	1
4	15	14	15	7
5	25	20	28	20
6	25	4	25	10
7	15	6	11	7
8	30	28	20	5
9	15	8	10	1
10	36	25	14	2
11	36	31	14	4
12	40	18	20	10
13	40	18	20	0
	307	193	207	71
	62% Survival		34% Survival	

as occur in the formation of medullary tube following ablation of the crest or the motor plate. These processes are much interfered with by the delaying action caused by the drug. It may be concluded, therefore, that in early neurula stages, sulfa treatment may be deleterious in a mechanical way by slowing down the healing and restorative processes — depending upon the type of operation. However, with all the factors considered, it is clear from table 2 that the percentage of

survival of sulfa treated embryos, when all groups are considered, is approximately twice as great as non treated embryos. The results of the tests show also that a dilution of 0.5% is as effective as a 1% solution.

In the 1945 operating season we (Detwiler and Robinson) obtained a high yield with sulfa treatment following unilateral excision of the brachial region of the cord from embryos in stages 21–25. These operations were done on embryos collected and carried from New Hampshire. Because of the lack of uniformity in the 1946 results given in table 1, where the type of operation and the stage varied considerably, we decided to repeat the 1945 operation upon different groups of eggs — thus keeping the type of operation constant. Eggs were used in order of availability from Nashville, Tenn., Durham, N. C., New Haven, Conn. and New Hampshire. The results are given in table 3. The beneficial effects of sulfa treatment are clearly indicated, but the percentage of survival (except for the New Hampshire eggs) was not as great as obtained in 1945. The higher survival of New Hampshire embryos in both 1945 and 1946 operating seasons may be indicative of a more hardy strain. On the other hand, the fact that these eggs were not shipped, but were transported personally under favorable conditions, may also be a factor favoring survival. From table 3 it is seen that in this particular instance, the postoperative mortality rate was highest among the eggs obtained from Durham. Only 18% of sulfa treated embryos survived as compared with 98% among the New Hampshire embryos. This is not meant to imply that New Hampshire eggs in general can be regarded as superior in quality to those from other regions. It merely suggests that the particular eggs gathered from the same pond in New Hampshire during the past two seasons have been hardy.

The experimental results upon eggs from Durham and New Haven became complicated because of contamination by ciliate protozoa. Whereas these protozoa apparently do not attack healthy unoperated embryos, there was evidence that they do aggregate and feed at open wound sites and increase

TABLE 3

Showing survival of embryos following unilateral removal of the brachial cord in Harrison's stages 23–25, and subsequent treatment as indicated.

SOURCE OF EGGS	NUMBER OF OPERATIONS	STAGE	HOURS IN SALT SOLUTION BEFORE TRANSFER	TREATMENT AFTER TRANSFER FROM SALT SOLUTION	NUMBER	SURVIVAL AFTER 12 DAYS	PER CENT SURVIVAL	REMARKS
Nashville	241	23–25	32	S.W.	110	19	17	
			14	S.D. (0.5–1.0%)	131	70	53	
Durham	150	23–25	18	S.W.	50	1	2	Contaminated with protozoa
				S.D. (0.5–1.0%)	100	18	18	
New Haven	140	24–25	24	S.W.	40	5	12.5	Contaminated with protozoa
				S.D. (0.5–1.0%)	59	26	44	
				S.D. (0.5) + Q.S. (1 mg/ml)	41	21	51	
New Hampshire	110	23–25	24	S.W.	16	9	43	
				S.D. (0.5–1.0%)	52	51	98	
				S.D. (0.5) + Q.S. (1 mg/ml)	42	40	95	

S.W. = spring water.
S.D. = sodium sulfadiazine.
Q.S. = quinine sulfate.

the mortality of the operated embryos. In instances where the concentration of protozoa was high, the mortality increased. Inasmuch as sodium sulfadiazine is not protozoacidal, we decided to experiment with quinine sulphate — a known protozoacide, in an endeavor to eliminate the ciliate organism without harm to the operated embryos. Using small dishes, 1 cm^3 of uniformly mixed protozoa culture was added to 1 cm^3

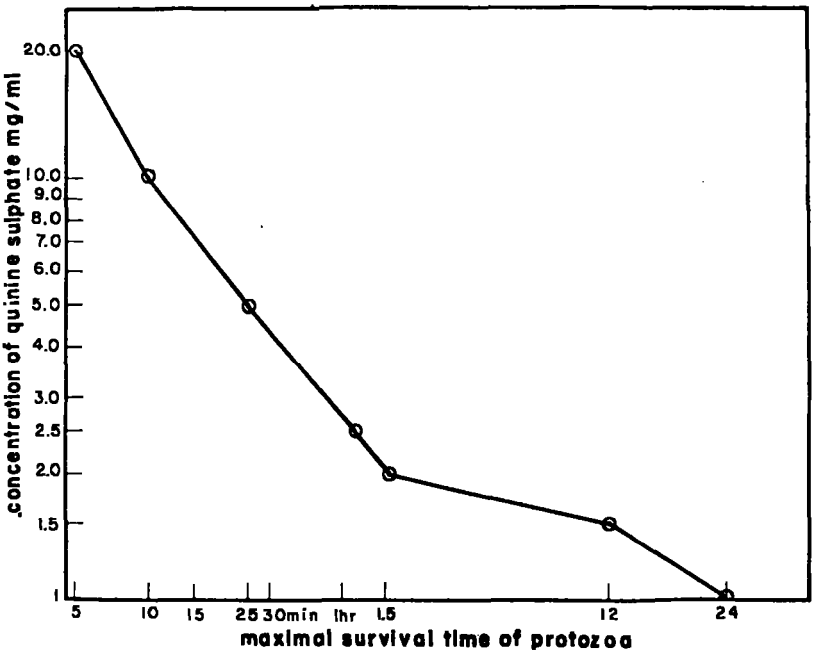

Fig. 1 Logarithmic plot showing relation between concentration of quinine sulphate and maximal survival of ciliate protozoa (see text).

of quinine sulfate solution in decreasing concentrations ranging from 20 mg/ml to 1 mg/ml as indicated in figure 1. The results showed that the higher concentrations, which killed off the protozoa in a very few minutes, were harmful to the embryos, but a dilution of 1 mg/ml which killed off all the protozoa in 1 cm^3 of culture in 24 hours was apparently not deleterious to the embryos in the stages employed (23–25). This concentration was employed, therefore, in one of two

sets of experiments where the egg clutches were contaminated with protozoa (New Haven material, v. table 3). The slightly higher survival of embryos following treatment with quinine sulfate in addition to sulfadiazine as compared with sulfa treatment alone tentatively suggests a beneficial effect of quinine sulfate in contaminated dishes. A check experiment to show that quinine sulfate in the concentrations used is not harmful to the operated embryos is shown in table 3. Operated embryos from New Hampshire eggs were divided into 3 groups and treated as follows: (a) spring water, (b) 0.5 to 1% sodium sulfadiazine and (c) 0.5 to 1% sodium sulfadiazine plus quinine sulfate of 1 mg/ml. The survivals of the embryos in combination c was as high as when sodium sulfadiazine alone was used. Although more experimentation is needed, the evidence thus far obtained indicates that treatment with a 1 mg/ml solution of quinine sulfate is not injurious to operated embryos and that this concentration will increase the number of surviving embryos when the latter are attacked by the protozoa.

OBSERVATIONS UPON SURVIVAL OF UNOPERATED EMBRYOS

This section deals with the effects of different concentrations of sodium sulfadiazine on normal embryos from different egg masses at several developmental stages. The solutions of sulfadiazine were made up in spring water in concentrations ranging from 0.125 to 2.0%. Embryos were placed in the solutions at the following stages: blastulae and gastrulae (Harrison's stages 8, 10, 12) and tail bud stages 25 and 28. In most instances, the embryos were reared in sulfadiazine until their controls in spring water had developed to stage 46 (the stage when feeding normally begins). This ranged from 17 to 21 days for the different groups. In several tests, the animals were kept in sulfadiazine for 2 months at which time they were nearing metamorphosis.

Data on the survival of embryos after 10 days in the different concentrations of sulfadiazine are shown graphically in figure 2. The embryos were divided into 6 groups according

to their source from different egg clutches, and each main group was subdivided according to the number of different solutions used. Each column on the graph represents the results for 20 embryos, and the height of each column shows

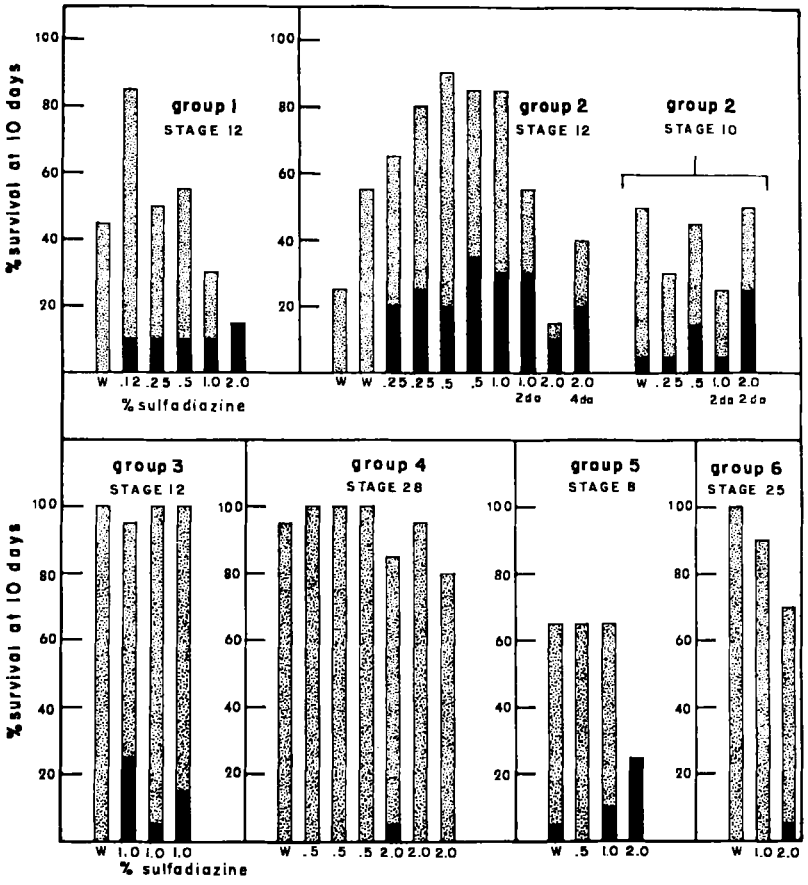

Fig. 2 Effect of sulfadiazine on survival of normal embryos. Each group of embryos came from a different egg clutch. Each column on the graph represents results for 20 embryos and the height of each column shows the percentage of total survivals at 10 days. The dotted portions of the columns indicate normal survivals and the solid portions show abnormal survivals, expressed as percentages of the total 20 embryos used in each test. With the exceptions noted on the graph, all embryos were in sulfadiazine or in spring water (W) for 10 days. In a few tests, group 2, they were transferred from sulfadiazine to water at 2 days and 4 days.

the percentage of total survivals. The solid portion of each column indicates the number of abnormal survivals expressed as a percentage of the total in each test.

In group 1 the observations were made upon embryos in yolk plug stages, and from a single egg clutch obtained from Durham, N. C. One sub-group of 20 embryos was reared in spring water and the other sub-groups were kept in the concentrations of sulfadiazine indicated. All embryos were reared at the same temperature and in the same quantities of solution; in other words, the difference in concentration of sulfadiazine was the only known environmental variable for the different subdivisions within any 1 group. In group 1, there were only 3 embryos (15%) surviving in the 2% solution of sulfadiazine after 10 days, and all of these were abnormal. They had developed to stage 37, but had small and abnormally shaped heads, weak heart contractions and no circulation. Controls in spring water were at stage 38 and had normal circulation. In the 1% solution, there were 6 embryos (30%) surviving at 10 days. Four of these were normal and 2 abnormal. The chief difference between the results for the 1% and 2% solutions in this group was as follows: in the latter all embryos were abnormal by 10 days and none survived to stage 46; in the former, a few, at least, survived to late larval stages when the observations were terminated. In the weaker solutions (0.12 to 0.5%) the percentage of survivals was much higher than in the 1% and 2% solutions, and the proportions of abnormalities was lower. For example, in a 0.12% solution in which 17 out of 20 animals (85%) survived there were 2 abnormal cases, representing 10% of the total or 11.7% of the survivals. In a 2% solution, the abnormalities at 10 days were only 15% of the total used for the test but 100% of the survivals. Most of the animals which failed to survive for 10 days in the 2% solution were abnormal early in the experiment. After 4 days in a 2% solution, 17 out of 20 embryos were surviving, but 13 were already abnormal.

In groups 1 and 2, there were fewer survivals in spring water than in weak concentrations of sulfadiazine. This would

seem to indicate that the bacteriostatic action of the drug is of value for unoperated embryos. Perhaps this effect becomes more apparent when normal embryos are reared together rather than in separate dishes. In groups 1 to 4 inclusive, 20 embryos were reared in each dish and the death and partial disintegration of an embryo within a common container increased the contamination for the other occupants. This was probably a factor in some of the tests although all dishes were examined twice daily for the removal of dead embryos and all solutions were changed at least 3 times during the 10-day period. In groups 5 and 6, each embryo was kept in a separate dish.

The embryos in group 2 came from New Haven material. Some of these were in yolk plug stages (stage 12); others were in early gastrula stages (stage 10). Survivals in group 2, stage 12, were somewhat more numerous than in group 1 but the same general result was noted as regards the greater toxicity of the higher concentrations. In a 2% solution, there were only a few survivals at 10 days and no survivals to stage 46. Better results were obtained in another test on stage 12 when the embryos were changed from 2% sulfadiazine to water after 4 days. In the latter case, 5 out of 20 embryos survived to stage 46. The results for a 1% solution in group 2, stage 12, were unexpected in that the embryos left in sulfadiazine for 10 days had a higher percentage of survivals than those which were changed to water at 2 days. However, the percentage of abnormalities in the latter test was already high when the transfer was made. Survivals for stage 10 of group 2 were less numerous than for the corresponding tests at stage 12. One of the 20 embryos of stage 10 developed abnormally in the control series, but this is expected occasionally for any group.

Embryos of group 3 were from New Haven material in which there was a 100% survival for the controls in spring water. In this group (stage 12 embryos) the percentage of survivals in a 1% solution was higher and the proportion of

abnormalities lower than in similar tests for groups 1 and 2. The results for group 5 gave further evidence for the view that the toxic effects of sulfadiazine differ markedly for different egg clutches. Embryos in group 5 were from New Hampshire material, and considering the fact that they were placed in sulfadiazine at stage 8 (earlier than any other groups) the percentage of survivals in a 1% solution was high.

Results for group 4 (New Haven material) and group 6 (New Hampshire material) showed that sulfadiazine had less effect on tail bud stage embryos than on the early stages. This difference was particularly striking for the 2% concentration. Whereas embryos of stages 8, 10 and 12 never survived beyond stage 46 in a 2% solution, a number of stage 25 embryos were reared for 8 weeks in this concentration. Their growth rate was retarded but there were few abnormalities.

Several types of abnormalities occurred when young embryos (stages 8–12) were exposed to sulfadiazine continuously. Circulation, which begins normally at stage 36, was often delayed until stages 38–39 and sometimes it was never established. There were 3 cases with double hearts out of a total of 260 embryos which were exposed either to a 1% or to a 2% concentration in the early stages. Twenty-three of the above total had spina bifida at the brachial level. In 45 embryos, an area of yolk resembling a large yolk plug remained uncovered at the anal region until relatively late embryonic stages. This condition occurred in all concentrations and occasionally even in water but it was most common in the 2% solution. In some cases, differentiation was inhibited generally and the embryos survived for several days as modified gastrulae. Other abnormalities observed occasionally were: reduction in number of gills, retarded development of the head, and partial cyclopia. Further details on abnormalities, with particular reference to the effects of sulfa drugs on embryonic blood development, will be taken up in a separate study at a later time.

CONCLUSIONS

The effects of sodium sulfadiazine (0.5–1.0%) upon Amblystoma embryos, following a variety of operations upon the central nervous system, are clearly indicated. In most instances the percentage of survival of sulfa treated embryos is considerably greater than the survival of embryos reared in water (tables 1 and 2). Exceptions to this are regarded tentatively as due to intolerance of certain embryos to the drug. This deduction is based upon observations with both unoperated and operated embryos. In general, early stages (blastula and gastrula) are more affected by the toxicity of the drug than are older stages (neural folds to tail bud). This difference is noted especially when the embryos are treated with a 2% solution; less so with 1%. For example, when unoperated embryos in early stages are reared in a 2% solution there is a high percentage of abnormalities and none survive beyond stage 46. When older embryos (stages 25–28) are reared in this concentration, the majority survive beyond stage 46 and many to late larval stages.

The observations upon operated embryos indicate that the action of a 0.5% solution of the drug is as effective as that of a 1% solution, and the former is preferable in that it produces few or no abnormalities when young stages are used.

Occasionally, egg clutches are contaminated with ciliate protozoa which increase the mortality rate of operated embryos. These protozoa can be destroyed within 24 hours with quinine sulphate in concentrations of 1 mg/ml without deleterious effects upon the embryos.

LITERATURE CITED

DETWILER, S. R., AND C. O. ROBINSON 1945 On the use of sodium sulfadiazine in surgery on amphibian embryos. Proc. Soc. Exp. Biol. and Med., *59:* 202–206.