Problems of Acetazolamide and N-Ethylnicotinamide as Teratogens

WALTER LANDAUER AND NOBORU WAKASUGI
Nuffield Institute of Comparative Medicine, The Zoological Society of London, Regent's Park, London NW 1

ABSTRACT Experiments with acetazolamide and N-ethylnicotinamide were made by injection into the yolk sac of chicken eggs after 24 and 96 hours of incubation. Acetazolamide produced in both stages abnormalities of the upper beak, the dosage-effect response following a linear course. Effects minor in incidence were rumplessness at 24 hours, bending of tibiotarsus shaft in both stages and muscular hypoplasia after 96 hours. N-ethylnicotinamide led to abnormalities of the upper beak in both stages. to malformations of tibiotarsus and fibula at 96 hours. With increasing dosages the results of N-ethylnicotinamide rose by threshold effects. In a particular strain of White Leghorn fowl embryos gave little or no response to acetazolamide; N-ethylnicotinamide produced the usual incidence of long bone defects, but few beak abnormalities. Acetazolamide acted as protective supplement when added to treatment with sulfanilamide; it prevented all abnormalities (micromelia) of long bones; it reduced incidence of defects of upper and lower beak after treatment at 24 hours, but not those of upper beak after 96 hours. N-ethylnicotinamide also gave complete protection against sulfanilamide-induced abnormality of lower beak and long bones, but an increased incidence of shortened upper beak. Acetazolamide and N-ethylnicotinamide injected together at 96 hours led to exaggeration in incidence and change in expressivity of defects of the upper beak and a drastic lowering in frequency of tibiotarsus-fibula malformations. Supplements of ADP reduced toxicity of acetazolamide and incidence of defects of the upper beak produced by it. Interference with NAD functions and oxidative phosphorylation is likely to be the cause of the teratogenic effects of acetazolamide as well as N-ethylnicotinamide.

Early in our studies dealing with the chemical induction of gross malformations of developing chicken embryos, we encountered the preventive or protecting effect of nicotinamide when given as supplement. A variety of compounds, such as insulin, physostigmine and sulfanilamide, which interfere with normal development of beak and limbs when they are injected into the yolk sac at appropriate stages of incubation, become innocuous in the presence of added nicotinamide (Landauer, '48, '49; Zwilling, '49). It was also noted, however, that above the protecting level nicotinamide tends to act itself as a teratogen. This is true, at the cost of high toxicity, when nicotinamide is the sole agent of interference, but occurs in a much more striking manner when it is used in certain combinations, a phenomenon to which we shall have occasion to revert hereafter. Our observations on the protective nature of nicotinamide led subsequently to experiments about the teratogenic activity of two analogs of nicotinamide, viz. 3-acetylpyridine and 6-aminonicotinamide (Landauer, '57, '65; Landauer and Clark, '62) and to tests on the combined effects of these analogs with sulfanilamide (Landauer and Clark, '64a, b).

Our present report will deal with two compounds, viz. N-ethylnicotinamide and sodium acetazolamide (2-acetylamino-1,3,4-thiadiazolo-5-sulfonamide; the Diamox of Lederle Laboratories). N-ethylnicotinamide, a derivative of nicotinic acid, was expected to be of interest in relation to our earlier work. The relationship of acetazolamide to our present undertaking will become clear as the evidence unfolds.

MATERIALS AND METHODS

We used for our experiments eggs from two separate interline crosses of White Leghorn fowl, bred by Thornbers Brothers Limited. The four pedigreed stocks which furnished the material for these crosses had varying histories of inbreeding. The crosses were designated as RM♀♀ × RA♂♂ and MB♀♀ × MD♂♂, respectively. Some data furnished by the Research Division of Thornbers suggest that the RM × RA cross

was slightly superior to the MB × MD cross in hatchability (though hatches of fertile eggs were in both crosses between 80 and 90%), in body weight at 23 weeks of age, in egg weight and in egg production. Any or none of these features may have a bearing on the results of our experiments. Eggs from the MB × MD cross were used only for special tests to which reference will be made; all our other experiments were pursued with eggs from the RM × RA cross. All eggs were incubated in a Petersime incubator with forced draft and automatic turning. Experimental treatment was done at 24 and, more commonly, at 96 hours of incubation.

Solutions of N-ethylnicotinamide (Aldrich Chemical Co.), sodium acetazolamide (Lederle Laboratories), 3-acetylpyridine (Sigma Chemical Co.) and nicotinamide (Hopkin and Williams, Ltd.) were made up in demineralized water, with 0.25% phenol added, and Tyndallized. Sulfanilamide (Sigma Chemical Co.) was handled in the same way, except that propylene glycol was the solvent. ATP and ADP (both C. F. Boehringer and Soehne) were dissolved in sterile water, which had been adjusted to pH 6.8 with phosphate buffer, and used within 2 to 3 hours.

For injections we used sterile, disposable tuberculin syringes and number 27 one-inch needles. Injections were made into the yolk sac; the hole, drilled for it into the shell, was covered with cellotape. The volume of solutions we used was 0.05 ml, except for a few tests in which 0.1 and 0.2 ml of acetazolamide was injected. All eggs were opened and the results recorded after 19 days of incubation, except in the case of one of the tests with ADP which for extraneous reasons was terminated after 16 days.

Among untreated embryos and in control tests in which known non-teratogenic compounds were used, the incidence of gross malformations was less than 1% of those surviving the seventeenth day of incubation and early mortality was very low. Except for the teratogen-specific results which are the subject of the present report, we found the same low incidence of morphological defects (listed as "miscellaneous" in the tables) in our experimental material. Standard errors of means, used throughout, were taken from the tables of Ritala ('33).

Gross malformations

The principal gross defect which treatment with acetazolamide produced at any time between 24 and 120 hours of incubation consisted in abnormalities of the upper beak. The most common abnormality was a slight or moderate shortening of the upper beak, but asymmetries (cross-beak) and, more rarely, clefts of the upper beak also occurred. Rumplessness, i.e., lack of tail vertebrae, was found after early injection of acetazolamide. Bending of the tibiotarsus shaft was encountered as a rare deviation. Muscular hypoplasia, particularly well-marked in the zeugopodial region, occurred as another rare malformation, and only following treatment with high doses of acetazolamide.

Experiments with N-ethylnicotinamide led to important defects in two separate parts of developing chicken embryos, viz., the upper beak and the zeugopodium. Shortening of the upper beak tended to be more extreme than after treatment with acetazolamide. This was especially true following injection of relatively large amounts of N-ethylnicotinamide and such cases were frequently associated with asymmetry (cross-beak) and sometimes with unilateral or bilateral clefts of the upper beak. Among long bones of the extremities, femur and tarsometatarsus were at times somewhat reduced in length, but tibiotarsus and fibula were often grossly abnormal. In the malformed condition the tibiotarsus was short and its distal head twisted laterally, thereby leading to luxation which forced the plantar surface of the foot away from the body. The fibula lacked a proximal head; in its stead the proximal end of the bone was fused with the tibiotarsus shaft below the lateral condyle of the tibia's proximal head. These abnormalities of the zeugopodium generally involved both legs to a similar extent, but asymmetries were not uncommon and, if present, the right tibiotarsus-fibula tended to be less abnormal than the left one or more rarely even escaped abnormality altogether. An occasional instance of rumplessness was found among embryos that had been

treated early, but this was not as frequent as after injection of acetazolamide.

Dosage and developmental stage

The effects of acetazolamide and N-ethylnicotinamide, according to dosage and stage of development at the time of treatment, are recorded in tables 1 to 4. A comparison of the results which acetazolamide had at 24 and 96 hours of incubation (tables 1 and 2) shows that at the latter stage of development the compound had relatively little toxicity, unless a very large amount (20 mg/egg) was used. Corresponding dosages were more toxic at 24 hours, but not in proportion to the much lesser weight of embryos at that age. It was noted, however, that acetazolamide was more toxic when the environmental temperature to which eggs had been exposed in transit and prior to incubation was relatively high; but this had little, if any, effect on incidence of malformations among survivors. Injection of 1 or 2 mg/egg acetazolamide at 96 hours did not interfere with normal development. Dosages between 2.5 and 10 mg/egg applied at 24 hours and between 5 and 20 mg/egg at 96 hours led to a rising incidence of shortened upper beak and this increase was nearly proportional to dosage, viz. from 15.9 to 51.9% after treatment at the early stage and from 18.7 to 73.6% following injection at the later period. There was no definite trend, on the other hand, in the effect which differing dosages of acetazolamide, given at 24 hours, had on incidence of rumplessness. The occurrence of unilateral bending of the tibiotarsus in one embryo which had been exposed to acetazolamide at 24 hours and of two that had been treated similarly at 96 hours is of interest in regard to results which will be reported and discussed later.

The results of our experiments with N-ethylnicotinamide (tables 3, 4) were in many ways quantitatively similar to those with acetazolamide. When 2.5 or 5 mg/egg of the compound were injected, N-ethylnicotinamide was rather innocuous to embryos of 96 hours, but, not unexpectedly, much more toxic to those of 24 hours of incubation. With a larger dose (10 mg/egg) N-ethylnicotinamide became too poisonous to make experimentation practical at 24 hours. With 2.5 and 5 mg/egg, given at 24 hours, N-ethylnicotinamide produced an incidence of 3.2 and 14.6%, respectively, of abnormalities of the upper beak. The extremities were not affected, nor did we observe the consistent involvement of other defects. Treatment at

TABLE 1

Effects of acetazolamide according to dosage and molarity. Treatment at 24 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

Dosage in mg (and μM)	2.5 11.25	5 22.50	10 45.00
Number treated	114	138	308
Mortality to 18 days, %	39.5	23.2	48.7
Survivors of seventeenth day	69	106	158
Short or abnormal upper beak, %	15.9	31.1	51.9
Tibia bent, %	0	0	0.6
Rumpless, %	18.8	14.2	20.9
Miscellaneous defects, %	0	0	0

TABLE 2

Effects of acetazolamide according to dosage and molarity. Treatment at 96 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

Dosage in mg (and μM)	1 4.50	2 9.0	5 22.5	10 45.0	20 90.0
Number treated	68	51	135	254	134
Mortality to 18 days, %	13.2	19.6	8.9	18.5	32.1
Survivors of seventeenth day	59	41	123	207	91
Short or abnormal upper beak, %	0	0	18.7	37.2	73.6
Miscellaneous defects, %	0	0	0	2.0	0

TABLE 3

Effects of N-ethylnicotinamide according to dosage and molarity. Treatment at 24 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

Dosage in mg (and μM)	2.5 16.77	5 33.53	10 67.07
Number treated	137	143	112
Mortality to 18 days, %	32.1	37.8	85.7
Survivors to seventeenth day	93	89	18
Short or abnormal upper beak, %	3.2	14.6	50.0
Rumpless, %	4.3	0	5.6
Miscellaneous defects, %	4.3	4.5	[1]

[1] Among the 18 survivors 5 had a short and abnormal tibiotarsus-fibula and 5 had muscular hypoplasia of the zeugopodium.

TABLE 4

Effects of N-ethylnicotinamide according to dosage and molarity. Treatment at 96 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

Dosage in mg (and μM)	2.5 16.77	5 33.53	10 67.07
Number treated	157	127	279
Mortality to 18 days, %	9.6	10.2	24.7
Survivors of seventeenth day	142	114	211
Short or abnormal upper beak, %	0.6	7.0	63.0
Short and abnormal tibiotarsus-fibula, %	7.7	59.6	74.4
Miscellaneous defects, %	1.4	0.9	0.5

96 hours of incubation led to a remarkable difference in the way in which the upper beak and tibiotarsus-fibula responded to the injection of increasing amounts of N-ethylnicotinamide. A doubling of dosage produced in each case a threshold effect and an abrupt rise in teratogenic activity, but the incidence of malformations of the upper beak lagged in the scale of dosages behind that for the zeugopodium.

Genotype and effect

We tested, for comparative purposes, the effects of acetazolamide and N-ethylnicotinamide on embryos derived from two separate interline crosses of White Leghorn fowl, viz. RM♀♀×RA♂♂ and MB♀♀×MD♂♂. The results are summarized in tables 5, 6. Experiments with 10 mg/egg acetazolamide at 96 hours (table 5) were performed on RM × RA embryos between January and early March and again in June. During these two test-periods we found among embryos that had survived the seventeenth day of incubation 34.7 and 34.0%, respectively, of individuals with a shortened or abnormal upper beak. In contradistinction, embryos of MB × MD origin and tested during the middle of March yielded no beak defects of any kind. To the latter embryos the dosage of 10 mg/egg at 96 hours was non-teratogenic or gave, in later trials, only minimal responses.

Comparative experiments with 10 mg/egg N-ethylnicotinamide at 96 hours (table 6) were performed on RM × RA embryos during January and Febuary and again during April and May, while tests with MB × MD embryos were conducted in March. The embryos of the two different sources of genetic origin reacted very similarly to what had been found with acetazolamide as far as the upper beak was concerned: RM × RA embryos produced during the two test-periods 56.0 and 71.6%, respectively, of individuals with abnormal upper beak, but the MB × MD embryos gave only 5.2% of such defects. The two periods when RM × RA embryos were tested did not produce statistically significant differences, but the effects obtained with MB × MD embryos differed in a highly significant way from either of the two results with RM × RA embryos. There was an almost total lack of response of MB × MD embryos to interference of beak development by N-ethylnicotinamide. In striking contrast, the two stocks gave no

TABLE 5

Tests with acetazolamide according to type of mating. 10 mg/egg 45.0 μM) injected at 96 hours of incubations

Matings and time of tests ('66)	RM♀♀ × RA♂♂ Jan.–March 3	MB♀♀ × MD♂♂ March 11–24	RM♀♀ × RA♂♂ June
Number treated	187	184	208
Mortality to 18 days, %	19.8	21.8	32.2
Survivors of seventeenth day	150	144	141
Short or abnormal upper beak, %	34.7±3.88	0	34.0±3.99
Miscellaeous defects, %	2.7	0	0

TABLE 6

Tests with N-ethylnicotinamide according to type of matings. 10 mg/egg (67.07 μM) injected at 96 hours of incubations

Matings and time of tests ('66)	RM♀♀ × RA♂♂ Jan.–Feb.	MB♀♀ × MD♂♂ March	RM♀♀ × RA♂♂ April–May
Number treated	159	126	120
Mortality to 18 days, %	31.4	23.0	15.0
Survivors of seventeenth day	109	97	102
Short or abnormal upper beak, %	56.0±4.75	5.2±2.26	71.6±4.47
Short and abnormal tibiotarsus-fibula, %	68.8±4.43	69.1±4.71	80.4±3.93
Miscellaneous defects, %	0	0	1.0

significant differences in the developmental response of the tibiotarsus-fibula to N-ethylnicotinamide.

Attempts to secure additional evidence relating to these genetic response differences were rendered impractical in the case of N-ethylnicotinamide on account of the excessive toxicity of amounts greater than those used in the previous tests (with 20 mg/egg at 96 hours post-operative mortality became 97.2%), but some supplementary information was obtained by treating MB × MD embryos with acetazolamide. These data refer to injecting 10 mg of the compound at 24 hours and either 10 or 20 mg at 96 hours of incubation. The results are given for survivors of the seventeenth day, and in parentheses of the fourteenth day, of incubation:

It appears from these data that doubling the amount of acetazolamide from 10 to 20 mg/egg injected at 96 hours of incubation did not overcome the nearly total lack of response of MB × MD embryos to the teratogen. The results of treatment at 24 hours, not previously done with MB × MD stock, suggest that at this early stage response to acetazolamide may not be quite as low as in the later stage, but a comparison with the data in table 3 indicates also that the reduced response of MB×MD embryos to acetazolamide involves the tail primordium as well as that of the upper beak.[1]

[1] It should be recalled that eggs from the two stocks were provided to us as available. We had no opportunity to analyze the genetic differences between these stocks.

	10 mg/egg 24 hours	10 mg/egg 96 hours	20 mg/egg 96 hours
Survivors seventeenth (fourteenth) day	27 (28)	39 (43)	49 (58)
Short or abnormal upper beak %	7.4 (10.7)	2.6 (4.7)	0 (3.4)
Rumpless %	3.7 (3.6)	—	—
Miscellaneous defects %	0	0	2.0 (1.7)

Sulfanilamide interactions

We conducted experiments at 24 and 96 hours of incubation in which the teratogenic activity of acetazolamide or N-ethylnicotinamide was observed in the simultaneous presence of sulfanilamide. The results are summarized in tables 7 to 9. The effects of 10 mg acetazolamide and of 1 mg sulfanilamide, given as single injections at 96 hours, produced for each of the two compounds the results characteristic of stage and dosage (table 7). When the two teratogens were used in combination, embryo mortality was reduced significantly below that which acetazolamide alone had caused (difference 19.9 ± 5.28%). The incidence of abnormalities of the upper beak was quite similar following treatment with acetazolamide alone or after supplementing it with sulfanilamide. All defects, on the other hand, which are specific for treatment with sulfanilamide and had occurred by injecting 1 mg of it, vanished with the presence of 10 mg acetazolamide. When we made similar tests with 24-hour embryos, but using only half the amount of acetazolamide, the results (table 8) showed again that all sulfanilamide-induced defects were forestalled by the presence of acetazolamide and that, in addition, a significant lowering in acetazolamide-induced shortening of the upper-beak occurred when sulfanilamide was present (difference 17.6 ± 6.31%).

In combination with N-ethylnicotinamide we found that sulfanilamide at 96 hours produced results (table 9) similar to what has just been described for supplementation of acetazolamide with sulfanilamide. As in the latter case, the defects typical of treatment with sulfanilamide, viz., shortening of the lower beak (with or without the secondary parrot beak feature) and micromelia were absent. Malformations of tibiotarsus and fibula occurred after the combined treatment in the same manner and with similar incidence as that observed after N-ethylnicotinamide alone. The frequency of production of a shortened upper beak was, however, significantly increased when treatment with sulfanilamide had been added to that with N-ethylnicotinamide (difference 24.1 ± 5.57%).

Nicotinamide and 3-acetylpyridine as supplements

When injection of 10 mg acetazolamide at 96 hours had been preceded four hours earlier by 2.5 mg nicotinamide (table 10), the incidence of abnormalities of the upper beak was significantly greater than after acetazolamide alone (difference 19.5 ± 6.33%). Five out of 105 embryos subjected to both compounds showed bends in the shaft of one of their tibiotarsi. Nicotinamide itself is non-teratogenic when given at the dosage level and stage used here, but larger amounts are shown to interfere with normal development of the upper beak. This will be discussed hereafter.

When 10 mg acetazolamide and 0.2 mg 3-acetylpyridine were given simultaneously to embryos of 96 hours of incubation (table 11), the incidence of abnormalities of the upper beak showed a suggestive rise. It is more important, however, that, compared with the incidence produced by 0.2 mg of 3-acetylpyridine alone, the combined treatment led to a highly significant in-

TABLE 7

Tests relating to the interaction of 10 mg/egg (45.0 μM) acetazolamide and 1 mg/egg (5.81μM) sulfanilamide injected at 96 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

Acetazolamide mg/egg	10	10	—
Sulfanilamide mg/egg	—	1	1
Number treated	163	168	171
Mortality to 18 days, %	50.3±3.91	30.4±3.55	12.3±2.52
Survivors of seventeenth day	81	117	150
Short or abnormal upper beak, %	40.7	41.9	0
Abnormal lower beak (short and parrot), %	0	0	32.7
Micromelia, %	0	0	23.3
Bent tibiotarsus (unilateral), %	1.2	0	0
Miscellaneous defects, %	0	0.9	0

TABLE 8

Tests relating to the interaction of 5 mg/egg (22.5 μM) acetazolamide and 1 mg/egg (5.81 μM) sulfanilamide injected at 24 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

Acetazolamide mg/egg	5	5	—
Sulfanilamide mg/egg	—	1	1
Number treated	143	148	135
Mortality to 18 days, %	42.0	50.7	31.1
Survivors of seventeenth day	83	73	93
Short or abnormal upper beak, %	31.3±5.10	13.7±3.77	1.1±1.08
Abnormal lower beak (short or parrot), %	0	0	33.3
Micromelia, %	0	0	30.1
Rumpless, %	20.5	12.3	1.1
Miscellaneous defects, %	0	1.4	1.1

TABLE 9

Tests relating to the interaction of 10 mg/egg (67.07 μM) N-ethylnicotinamide and 1 mg/egg (5.81 μM) sulfanilamide injected at 96 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

N-ethylnicotinamide mg/egg	10	10	—
Sulfanilamide mg/egg	—	1	1
Number treated	112	110	106
Mortality to 18 days, %	11.6	22.7	11.3
Survivors of seventeenth day	99	85	94
Short or abnormal upper beak, %	67.7±4.71	91.8±2.98	0
Abnormal lower beak (short or parrot), %	0	1.2	33.0
Micromelia, %	0	0	21.3
Short and abnormal tibiotarsus-fibula, %	78.8	65.9	0
Miscellaneous defects, %	0	0	0

TABLE 10

The effect of injecting 2.5 mg/egg (20.47 μM) nicotinamide at 92 hours of incubation, followed four hours later by 10 mg/egg (44.50 μM) acetazolamide and of acetazolamide alone at 96 hours. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

	Nicotinamide, 2.5 mg/92 hours Acetazolamide, 10 mg/96 hours	Acetazolamide, 10 mg/96 hours
Number treated	137	193
Mortality to 18 days, %	23.4	26.9
Survivors of seventeenth day	105	141
Short or abnormal upper beak, %	57.1±4.83	37.6±4.09
Bent tibia (unilateral), %	4.8	0
Miscellaneous defects, %	0	0

TABLE 11

Tests relating to the interaction of 10 mg/egg (45.0 μM) acetazolamide and of 0.2 mg/egg (1.65 μM) 3-acetylpyridine at 96 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

Acetazolamide mg/egg	10	10	—
3-acetylpyridine mg/egg	—	0.2	0.2
Number treated	101	95	84
Mortality to 18 days, %	26.7	33.7	14.3
Survivors of seventeenth day	74	63	72
Short or abnormal upper beak, %	39.2	49.2	1.6
Muscular hypoplasia, %	0	31.7±5.86	8.3±3.26
Miscellaneous defects, %	1.4	3.2	0

crease in the frequency of muscular hypoplasia (difference 23.4 ± 6.71%). The combination of N-ethylnicotinamide and 3-acetylpyridine, tested at 96 hours of incubation, had no noticeable effect on incidence of muscular hypoplasia or on other symptoms.

Combined activity of acetazolamide and N-ethylnicotinamide

In view of the phenotypic similarity which acetazolamide and N-ethylnicotinamide produce in development of the upper beak and because of the homologous reactions of the two teratogens in combination with sulfanilamide, it seemed of interest to study any interactions that might occur in their simultaneous administration. Such tests, using 5 mg/egg dosages of each of the two compounds, were performed at 96 hours of incubation and their results are presented in table 12. Post-operative mortality did not differ greatly between the three experimental groups, that is, acetazolamide and N-ethylnicotinamide separately or combined. The incidence of all kinds of defects of the upper beak was 5.7 ± 2.28% after 5 mg N-ethylnicotinamide, 19.7 ± 3.62% after 5 mg acetazolamide, and 66.4 ± 4.47% following treatment with both teratogens.

The data of table 13 afford a comparison of total incidence of defects of the upper beak and its breakdown into three sub-groups, as they were observed by treatment with either 5 or 10 mg acetazolamide or N-ethylnicotinamide, respectively, or with the combined injection of 5 mg of each of the two compounds. It can be seen that the total incidence of abnormalities of the upper beak, following combined treatment, was very similar to that produced by 10 mg of N-ethylnicotinamide, but much greater than that after 10 mg acetazolamide (difference 29.2 ± 5.75%). As shown earlier, there was within the range of the present tests a direct dosage-effect relationship for acetazolamide, whereas dosage above 5 mg/egg N-ethylnicotinamide led to a threshold with sudden rise of teratogenicity beyond it. Our results indicate that such a threshold for defects of the upper beak occurs with acetazolamide if applied in combination with other teratogens, such as sulfanilamide or 3-acetylpyridine, and with N-ethylnicotinamide in the presence of acetazolamide. Beyond this quantitative cause-effect relationship, the data demonstrate that a qualitative shift had taken place in the spectrum of malformations of the upper beak: the combined treatment of acetazolamide and N-ethylnicotinamide led to a relative lowering in incidence of shortening of the upper beak, to a rise in that of cross-beak, and to a great increase in the production of clefts of the upper beak.

Another result of great interest concerns the effect which the combined administration of acetazolamide and N-ethylnicotinamide had on abnormality of the tibiotarsus-fibula. It will be remembered that acetazolamide did not, with any dosages tested, interfere with normal development of the long bones, except for the rare occurrence of a bent in the tibiotarsus shaft. Treatment with 5 mg N-ethylnicotinamide produced a 40.6% incidence of abnormality of the tibiotarsus-fibula and this rose to 74.4% with a dosage of 10 mg. The combined treatment, however, yielded an incidence of only 15.9% of this defect. It must be concluded that the presence of acetazolamide led in this respect to a highly significant lowering (24.7 ± 5.89%) of the teratogenic activity of N-ethylnicotinamide.

Supplementation with ATP and ADP

When 2.5 mg/egg ATP were used as supplement to 10 mg/egg acetazolamide or N-ethylnicotinamide, injected at 96 hours, a suggestive lowering of embryo mortality occurred, but neither combination produced significant changes in incidence or expressivity of the deviations due to the unsupplemented teratogens.

The results of analogous tests with ADP are presented in table 14. It can be seen that 2.5 mg/egg of ADP added to treatment with 10 mg acetazolamide led to a suggestive lowering of post-operative embryo mortality, that it increased significantly the number of embryos which escaped harm (difference 18.0 ± 5.04%) and reduced significantly the incidence of defects of the upper beak (difference 17.2 ± 5.01%).

Supplementation of 10 mg N-ethylnicotinamide with 2.5 mg ADP at 96 hours re-

TABLE 12

Tests relating to the interaction of 5 mg/egg (22.5 μM) acetazolamide and of 5 mg/egg (33.53 μM) N-ethylnicotinamide injected at 96 hours of incubation. Embryos from matings of RM♀♀ × RA♂♂ White Leghorn fowl

Acetazolamide mg/egg	5	5	—
N-ethylnicotinamide mg/egg	—	5	5
Number treated	154	157	143
Mortality to 18 days, %	20.8	28.0	25.9
Survivors of seventeenth day	122	113	106
Short or abnormal upper beak, %	19.7±3.62	66.4±4.47	5.7±2.28
Short and abnormal tibiotarsus-fibula, %	0	15.9±3.40	40.6±4.81
Miscellaneous defects, %	0.8	0	0

TABLE 13

Details of defects of the upper beak induced by combined treatment of 5 mg/egg each of acetazolamide and N-ethylnicotinamide (as reported in table 12) and their comparison with results, produced separately, by 5 or 10 mg/egg, respectively, of acetazolamide and N-ethylnicotinamide.

Dosage in mg/egg at 96 hours	Acetazolamide		Acetazolamide and N-ethylnicotinamide	N-ethylnicotinamide	
	10	5	5 each	5	10
Short upper beak, %	27.1	14.8	13.3	2.8	34.6
Cross-beak, %	9.7	4.9	36.3	1.9	28.0
Clefts of upper beak, %	0.5	0	16.8	0.9	0.5
All defects of upper beak, %	37.2	19.7	66.4	5.7	63.0

TABLE 14

The effects of acetazolamide and N-ethylnicotinamide alone or in combination with ADP. ADP was injected just prior to one of the two teratogens. The embryos treated with N-ethylnicotinamide, alone or combined with ADP, were examined after 16 days of incubation, the others after the regular 19-day period.

	Acetazolamide 10 mg	ADP 2.5 mg Acetazolamide 10 mg	N-ethyl-nicotinamide 10 mg	ADP 2.5 mg N-ethyl-nicotinamide 10 mg
Treated	148	151	146	145
Mortality to eighteenth day, %	18.9	11.9	19.9	12.4
Survivors of seventeenth day	120	133	117	127
Normal, %	70.0±4.18	88.0±2.82	31.6	46.5
Abnormal upper beak, %	29.2±4.15	12.0±2.82	38.5	30.7
Tibia bent, %	1.7	0	—	—
Abnormal tibiotarsus-fibula, %	—	—	50.4	37.8
Miscellaneous defects, %	0	0	0	1.6

duced post-operative mortality in a manner similar to that observed in connection with acetazolamide. There was a suggestive increase in the incidence of embryos which remained normal (difference 15.9 ± 6.18%) and there were equally suggestive decreases in the frequencies of abnormalities of upper beak and of tibiotarsus and fibula, but these changes failed to come into the range of true statistical significance.

DISCUSSION

N-ethylnicotinamide has apparently not been tested in any organism for its teratogenic activity, but in experiments with mice the compound was found to be almost as effective as nicotinamide as a precursor of liver NAD (Kaplan, Goldin, Humphreys and Stolzenbach, '57). It does not seem to be known, however, if the NAD thus substituted would function normally either by conversion into nicotin-

amide or otherwise. Acetazolamide was at first reported to be non-teratogenic, when injected parenterally into pregnant rats and rabbits (Lutwak-Mann, '55; Hay, Adams and Lutwak-Mann, '60), but Layton and Hallesy ('65) demonstrated convincingly that the compound produces deformities of the right forelimb of newborn rats whose mothers had received it with their feed. These malformations consisted in the absence of one or more toes, and in more severe cases also involved bones of the forearm. The findings of Layton and Hallesy were confirmed by Wilson, Maren and Takano ('66). Both groups of investigators assumed that the teratogenic activity of acetazolamide was due to inhibition of carbonic anhydrase. In experiments with chicken embryos (developmental stage, dosage and route of administration not given) Shepard ('62) had been unable to produce malformations with acetazolamide, a failure that may have its explanation in our finding that response depends upon genotype.

Our present observations have shown that N-ethylnicotinamide and acetazolamide, when injected into incubating eggs, produced among the embryos interesting similarities as well as differences of morphological response. Both compounds interfered with normal formation of the upper beak. Acetazolamide did so, at 24 and 96 hours of incubation, with a strictly linear dosage-effect relationship, slight degrees of shortening after small doses being superseded by more extreme reduction and more frequent asymmetry (cross-beak) with larger amounts. With N-ethylnicotinamide, in contrast, increasing doses of the compound led at both developmental stages to spurts of sudden rise in the incidence of beak defects. Extent of abnormality of the upper beak tended to be somewhat more extreme after treatment with N-ethylnicotinamide than after that with acetazolamide.

With acetazolamide as well as with N-ethylnicotinamide incidence of abnormalities of the upper beak was higher following treatment at 24 than at 96 hours of incubation. Dosage relative to embryo weight was, of course, greater at the earlier stage, but the occurrence of *any* beak abnormality had been unexpected after the early treatment. For, it had been our general experience that teratogens do not at the 24-hour stage interfere with normal development of the upper beak, a rule to which in previous work we had as only exception encountered the effect of relatively large doses of nicotinamide.

When applied at 24 hours of incubation acetazolamide as well as N-ethylnicotinamide led in some instances to supression of tail development — more often in the case of acetazolamide than after N-ethylnicotinamide — but there was no discernible relation of dosage to effect, except that, as related earlier, embryos of MB × MD stock gave a much reduced incidence of rumplessness when treated with acetazolamide at 24 hours of incubation.

Treatment with acetazolamide produced abnormalities of the long bones only in very rare instances and these were confined to a bend in the shaft of the tibiotarsus (tables 1, 7, 14). Such defects of the tibiotarsus seemed to occur somewhat more often after combined treatment with acetazolamide and nicotinamide (table 9). N-ethylnicotinamide, when injected at 24 hours of incubation, never interfered with normal development of the extremities. Treatment with N-ethylnicotinamide at 96 hours, on the other hand, produced the specific malformations of tibiotarsus and fibula on which we have reported. The data in table 4 indicate that above treatment with 2.5 mg/egg (incidence 7.7%) there is a threshold of greatly increased response, leading to an incidence of tibiotarsus-fibula defects of 59.6 and 74.4% after 5 and 10 mg/egg, respectively. It should be noted (table 4) that similar levels of incidence occurred for the tibiotarsus-fibula defects one step earlier in the rising scale of dosages than with abnormalities of the upper beak.

Parallelisms between acetazolamide and N-ethylnicotinamide were, as in other reactions, found in their response to combined treatment with sulfanilamide, nicotinamide or 3-acetylpyridine, and this will be discussed below.

Why N-ethylnicotinamide should interfere specifically with the skeletal elements of the zeugopodium remains unknown. The fact that in these deviations from normal development the fibula suffers more

than the tibiotarsus may find its explanation in the competition for sources of mesenchyme which Wolff and Hampé ('54), Hampé ('59, '60) and Wolff and Kieny ('62) found in normal formation of these skeletal elements and, in a greatly exaggerated manner, after surgical excision of mesenchyme or after exposure of leg buds to x-rays.

The dissimilarities between embryos of the RM × RA and the MB × MD stocks in their reactions to the teratogens used in our tests raise two questions of considerable interest: Are these differences in response related to stage or dosage or are they of a qualitative biochemical order? And, secondly, what is the nature of the dissociation of symptoms which N-ethylnicotinamide produces in MB × MD embryos, compared to those of RM × RA stock? Since the gradient of response to increasing amounts of N-ethylnicotinamide is steeper in the zeugopodial skeleton than in development of the upper beak, we looked for additional quantitative information in tests with MB × MD embryos. N-ethylnicotinamide was found to be too toxic above the dosages used in our earlier tests at 24 and 96 hours of incubation (tables 3, 4). In experiments with acetazolamide we could show, on the other hand, that using a greatly increased dose of the compound produced little, if any, augmentation of interference with normal development, and further that treatment of embryos at 24 hours gave this low reactivity not only in the primordia of the upper beak, but in those of the tail skeleton as well. It appears, therefore, that qualitative features, operated in the case of RM × RA embryos by a threshold mechanism, distinguish the two stocks in their response or lack of response. The nature of these dissimilarities remains unknown. Threshold effects of a kind similar to the present ones are well recognized features of gene expression or suppression, e.g., in the presence or absence of the ventral tubercles of cervical vertebrae of mice (McNutt, '54).

Embryos of the MB × MD stock pose another fascinating problem in that, following treatment at 96 hours of incubation, their morphogenetic response in tibiotarsus-fibula development is as great as that found among RM × RA embryos whereas that of the upper beak is drastically reduced. Response differences of organs or parts to specific teratogens have been encountered frequently in the presence of dissimilarity of genotype, e.g., in chicken embryos (review Landauer, '54) and mice (Kalter and Warkany, '57; Dagg, '64–65; Dagg, Schlager and Doerr, '66). Such evidence came from experimentation with *one* teratogen, often tested at different levels of dosage or developmental stage. Our present results are of interest because they deal with *two* teratogens. If only one of the teratogens, viz. acetazolamide, had been used, we should have concluded that the differences in response of embryos of the two stock are a universal reflection of their respective genotypes. Our tests with N-ethylnicotinamide showed, however, that in the presence of two different genotypes one part or organ (the upper beak) may greatly differ in response and another one (tibiotarsus-fibula) react in virtually identical fashion to variations of residual heredity. Such dissociations of "syndromes" are known to occur in the manifestations of specific genes, e.g., among fowl in the Po^d gene for polydactylism in which variations in expression of the polydactyl conditions of fingers and toes may diversely be combined with a radius that is either absent, normal or doubled (Landauer, '56) or among man in the varied manifestations of the mutant genes for osteopsathyrosis (Bell, '28; Ottley, '32) or for ectodermal dysplasia (Clouston, '29, '39). The curious dissociation of symptoms which N-ethylnicotinamide produces in MB × MD embryos, or their combination in RM × RA embryos, present presumably additional evidence for the conclusion that the organ-specific responses are qualitative rather than quantitative in nature.[2]

As mentioned previously, Layton and Hallesy ('65) as well as Wilson, Maren and Takano ('66) suggested in their re-

[2] An interesting hereditary condition of chicken embryos, with an important bearing on the nature of dissociable syndromes, has just been reported by U. K. Abbott and J. A. MacCabe (Ectrodactyly: A new embryonic lethal mutation in the chicken. J. Hered., 57: 207–211, '66). They described a recessive gene substitution of chicks which is responsible for abnormal development of the upper beak, but which leads, in addition, to striking defects of the extremities if it occurs in individuals who are also homozygous for the mutant "scaleless" in which development of feathers and scales is severely inhibited.

ports that the appearance of skeletal defects among newborn rats, whose mothers had during pregnancy been given acetazolamide in their feed, was the result of inhibition of carbonic anhydrase. There seems to be no evidence for this conclusion, except the fact that inhibition of carbonic anhydrase is the best known and apparently most specific activity of the compound. It would, however, seem unusual for carbonic anhydrase inhibition to produce teratogenic effects in any part of the skeleton since little or no activity of the enzyme has been discovered in unossified cartilage of either chickens (Dulce, Siegmund, Körber and Schütte, '60) or rats (Ellison, '65).[3] Negative evidence of either kind is obviously inconclusive. But it must also be kept in mind that acetazolamide, presumably like all inhibitors of enzymes, is not entirely specific (Davenport, '62), and that it has been found to produce effects on the activity of other enzymes, e.g., phosphatase (Siegmund and Dulce, '60).

It seemed to us that evidence concerning the inhibition of carbonic anhydrase as teratogenic agent might be found by using another inhibitor of the enzyme in combination with acetazolamide and observing the occurrence of additive effects. For our experimental requirements (solubility in a non-toxic vehicle and relatively low toxicity of the inhibitor) sulfanilamide seemed the compound of choice, even though its role as inhibitor of carbonic anhydrase was, compared with acetazolamide, known to be low (Maren, Mayer and Wadsworth, '54; Jukes and Broquist, '63). The combined effect of the two presumed inhibitors (tables 7, 8) proved interesting and unexpected in that the presence of acetazolamide completely abolished the teratogenic effects of sulfanilamide, while sulfanilamide did nothing to interfere, in a positive or negative way, with the quantitative or qualitative results of acetazolamide administration. Neither of these results can well be interpreted as adjuvant or conjoint effects of carbonic anhydrase inhibition.

We decided to study next the results of combining the administration of N-ethylnicotinamide with sulfanilamide (table 9). The reasons were twofold. We wished to ascertain if the similarity in activity and response of acetazolamide and N-ethylnicotinamide extended to interaction with sulfanilamide. Secondly, since it had been reported that N,N-diethylnicotinamide has an appreciable diuretic effect (Boyd and Forde, '40) and since it has apparently not been determined if the monoethylnicotinamide, used in our experiments, produces similar results, it seemed worthwhile to make tests on the assumption that the two compounds act similarly. For analogous reasons we inquired subsequently into the results of combined treatment with acetazolamide and N-ethylnicotinamide.

As with acetazolamide, the effects of sulfanilamide were completely (micromelia) or nearly completely (short lower beak) prevented in the presence of N-ethylnicotinamide. The incidence of one of the N-ethylnicotinamide-specific malformations, viz. defective formation of tibiotarsus-fibula, was not altered in the presence of sulfanilamide, but that of the other one, viz. shortening or defectiveness of the upper beak, was greatly increased (difference 24.1 $\pm$ 5.57%). The similarity in activity of acetazolamide and N-ethylnicotinamide thus received new support, but again there is no evidence of likelihood of this consimilarity being based on inhibition of carbonic anhydrase. The meaning of exaggeration in incidence of abnormalities of the upper beak will be commented upon below.

The results of combined treatment with acetazolamide and N-ethylnicotinamide were reported in some detail in tables 12 and 13. It is of interest to note that the activity of acetazolamide as inhibitor of carbonic anhydrase is not reversed by the addition of p-aminobenzoic acid (Maren, Mayer and Wadsworth, '54). The same was found to be true for the teratogenic activity of sulfanilamide, but it was learned that the occurence of parrot beak and micromelia, due to treatment with sulfanilamide, can be prevented by giving nicotin-

[3] It should be noted also that the acetazolamide-induced abnormalities of the upper beak can be produced prior to the appearance of erythrocytes or other tissues with known carbonic anhydrase activity, viz., by treatment at 24 hours of incubation. It is possible, of course, as appears to be true for sulfanilamide (Zwilling and DeBell, '50), that acetazolamide does not become incorporated into embryonic tissues until relatively late in development.

amide as supplement (Zwilling and DeBell, '50). Acetazolamide in similarly protecting against sulfanilamide-induced abnormality of the lower beak and of the legs thus appears to have nicotinamide or NAD-like properties. The same is true for N-ethylnicotinamide. Yet more surprisingly, acetazolamide has a notable effect in reducing incidence and extent of the malformations of tibiotarsus-fibula brought about by treatment with N-ethylnicotinamide.

In contrast to the enhanced normality of the zeugopodium, we found that the combined treatment of acetazolamide and N-ethylnicotinamide greatly exaggerated the occurrence of a shortened or abnormal upper beak. This phenomenon is of peculiar interest and has a long history of comparable observations. In experiments with physostigmine sulfate (1 mg/egg 96 hours) it was found (Landauer, '49) that supplements of nicotinamide (5 mg/egg) prevented the occurrence of micromelia, syndactylism and parrot beak, but produced a rise in the incidence of shortened upper beak from 2.1 to 27.6%. In a similar way, Zwilling and DeBell ('50) reported that micromelia and parrot beak caused by treatment with sulfanilamide were completely prevented by the addition of nicotinamide, but that in their place shortening of the upper beak had occurred with an incidence of 49.4%; after treatment with sulfanilamide alone the frequency of the same abnormality had been only 1.17%. Landauer and Clark ('64a) observed that in the combination of 3-acetylpyridine and sulfanilamide, administered at 120 hours of incubation, symptoms of teratogenicity due to sulfanilamide were virtually abolished, while the effects of 3-acetylpyridine, including shortening of the upper beak, were greatly exaggerated in incidence. A very similar situation obtained when 6-aminonicotinamide and 3-acetylpyridine were used in combination (Landauer and Clark, '64b). We know from extensive earlier observations that the injection of small amounts (5 mg/egg) of nicotinamide at 96 hours of incubation may, as sole teratogenic effect, produce a low incidence of shortened upper beak and that the same kind of abnormality (and none other) occurs after increased (and rather toxic) dosage or after application in earlier stages of development. It seems likely that in the presence of such compounds as physostigimine and sulfanilamide a threshold is passed for heightened teratogenic activity of nicotinamide and that sulfanilamide has the same effect on N-ethylnicotinamide. Another clear instance of synergism was found in the striking rise in muscular hypoplasia that occurred after combined treatment of acetazolamide and 3-acetylpyridine. These synergistic effects are clearly of great importance in understanding the metabolic activity of acetazolamide and N-ethylnicotinamide in our material and their role as teratogens.

Two general conclusions seemed warranted by our observations on the combined activity of acetazolamide and N-ethylnicotinamide and on the interaction of either of them with sulfanilamide, 3-acetylpyridine and nicotinamide, viz. that competition for specific sites of developmental interference plays an important role in the morphogenetic expressions to which they lead and that the evidence of synergism demonstrates the existence of dissimilarities in steps of shared metabolic pathways. The important role which competition plays in interference with development and hence in the origin of abnormality has been discussed in previous reports (Landauer and Clark, '62, '64a) and need not be examined again.

The fact that we found in our experiments neither additive effects nor evidence of synergism when we combined treatment with two known inhibitors of carbonic anhydrase, viz. acetazolamide and sulfanilamide, pointed to the likelihood that another mechanism accounts for the teratogenic activity of either of the two compounds. As far as sulfanilamide was concerned, we proceeded earlier on the basis of the well-established observation that the nicotinamide analog 3-acetylpyridine, when substituted for nicotinamide, fulfills some of the normal functions of NAD and were, on that basis, successful in using the analog in place of nicotinamide to protect developing chicken embryos against the teratogenic activity of sulfanilamide (Landauer and Clark, '64a). Our present observations showed (1) that acetazolamide resembles 3-acetylpyridine

in its usefulness to forestall the teratogenic effects of sulfanilamide and that the same is true for N-ethylnicotinamide; (2) that in combination with N-ethylnicotinamide, as in that with nicotinamide itself, sulfanilamide leads to a high degree of synergism in the incidence of abnormal development of the upper beak. The combination of acetazolamide and N-ethylnicotinamide produced both types of effects: protection against N-ethylnicotinamide-induced abnormalities of tibiotarsus-fibula by supplementation with acetazolamide and, one the other hand, exaggeration in the incidence of defects of the upper beak. Our evidence of synergism clearly is proof that interference had taken place in dissimilar steps of a shared enzymatic process. Many of our observations have pointed to the conclusion that we were dealing with an inhibition of metabolic events related to NAD and oxidative phosphorylation. The effect of acetazolamide in protecting against the teratogenicity of sulfanilamide can not, however, (as may have been true in our experiments with 3-acetylpyridine; Landauer and Clark, '64a) have been due to conversion into nicotinamide.

These observations and conclusions led us to tests with ATP and ADP. Supplementing the treatment of either acetazolamide or N-ethylnicotinamide with ATP had not significant effect on the teratogenic activity of the two compounds, perhaps because of improper timing, inadequate dosage or loss of activity. Additions of ADP, however, greatly reduced toxicity and teratogenicity of acetazolamide, thereby providing important support for our hypothesis that the teratogenic effects of acetazolamide are in our material brought about by interference, at one point or another, with the synthesis or functions of NAD. Much of our evidence suggests that the same is true for the teratogenic activity of N-ethylnicotinamide, but further experimentation will be required on this question.

Finally, two points seem worthy of special emphasis, viz., how well our material illustrates the principle that in teratological events particular organs and the individual cells of which they are composed are genetically determined, yet depend for survival and for realization of their destiny on factors of the internal environment and the products of its metabolic activity, and secondly, how clearly our evidence demonstrates the early segregation of genetically determined tissue responses.

SUMMARY

The effects of acetazolamide and N-ethylnicotinamide were studied on developing chicken embryos by injecting given amounts into the yolk sac. The principal results, as recorded on survivors of the seventeenth day of incubation, were as follows:

1. The main effect of acetazolamide, following treatment at either 24 or 96 hours of incubation, consisted in abnormalities of the upper beak, viz. a shortened or crossed condition or, much less commonly, marginal clefts. Some incidence of rumplessness was found after treatment at 24 hours. In rare instances bending of the tibiotarsus shaft was observed after treatment at either stage and muscular hypoplasia as a result of treatment at 96 hours.

2. The deviations from normal development of the upper beak, produced by acetazolamide, followed in their incidence a linear dosage-effect relationship at both stages. At the 24-hour stage dosages between 2.5 and 10 mg/egg resulted in an incidence of 15.9 to 51.9% of malformations of the upper beak; at the 96-hour stage a range of 5 to 20 mg/egg led to the occurrence of 18.7 to 73.6% of similar defects.

3. The principal effects of N-ethylnicotinamide, common to treatment at 24 and 96 hours, were abnormalities of the upper beak, resembling those produced by acetazolamide. Treatment at 24 hours did not interfere with normal formation of the extremities, but when injected at 96 hours N-ethylnicotinamide led to striking malformations of tibiotarsus and fibula, often associated with some shortening of femur and tarsometatarsus.

4. N-ethylnicotinamide produced with advancing dosage of injection sudden steps of incerasing incidence of malformations. These threshold effects occurred at the 24-hour stage for defects of the upper beak and at the 96-hour stage for abnormalities of both upper beak and zeugopodium.

5. Treated with equivalent amounts, acetazolamide and N-ethylnicotinamide both tended to produce a higher incidence of beak defects at 24 than at 96 hours.

6. In comparing embryos from two different interline crosses of White Leghorn fowl it was found that in embryos of one of these crosses, irrespective of dosage and stage, acetazolamide had little or no teratogenic effect and that treatment with N-ethylnicotinamide interfered little, if at all, with normal beak development, yet led in embryos of both crosses to a similar incidence of malformations of tibiotarsus and fibula.

7. When embryos were simultaneously treated with acetazolamide and sulfanilamide at the 24-hour stage the usual symptoms of sulfanilamide teratogenicity (short lower and parrot beak, micromelia) did not occur and the incidence of abnormalities of the upper beak was reduced. The same protection against sulfanilamide occurred at 96 hours, but the incidence of acetazolamide-induced defects of the upper beak remained unchanged.

8. Tests at 96 hours with the combined treatment of N-ethylnicotinamide and sulfanilamide gave, as in the presence of acetazolamide, complete protection from damage by sulfanilamide, but the incidence of abnormal upper beak was significantly raised.

9. When nicotinamide was given at 92 hours and acetazolamide 4 hours later, the incidence of abnormal upper beak was significantly increased.

10. When 3-acetylpyridine and acetazolamide were injected simultaneously at 96 hours, the incidence of muscular hypoplasia was greatly increased.

11. The combination of acetazolamide and N-ethylnicotinamide, applied at 96 hours, produced an exaggeration in incidence and a shift in expressivity of abnormalities of the upper beak, but also a drastic lowering in the frequency with which abnormalities of the tibiotarsus-fibula were produced.

12. Supplementing acetazolamide with ADP lowered post-operative mortality; it increased significantly the frequency of embryos developing normally by greatly lowering the incidence of abnormalities of the upper beak. Supplementing N-ethylnicotinamide with ADP had effects that tended in the same direction, but without reaching clear-cut levels of statistical significance.

13. Competition for specific sites appears to play an important role in the origin of the developmental defects produced by acetazolamide and N-ethylnicotinamide.

14. It seems unlikely that the teratogenic effect of acetazolamide on chick development is mediated *via* inhibition of carbonic anhydrase.

15. Cumulative evidence points to the conclusion that both acetazolamide and N-ethylnicotinamide interfere with NAD functions and oxidative phosphorylation and, thereby, with normal development.

ACKNOWLEDGMENTS

The possibility of pursuing the present studies was in first place due to the cordial hospitality of Dr. L. G. Goodwin, Director of the Nuffield Institute of Comparative Medicine and to the interested and generous co-operation of Dr. John C. Bowman, Chief Geneticist in the Research Division of Thornber Brothers Limited in Mytholmroyd (Halifax, Yorkshire). About half of the acetazolamide used in our experiments was provided by Dr. A. T. Mennie and Lederle Laboratories Division, Cyanamid of Great Britain Limited. Various phases of our work had the liberal support of grants from the National Science Foundation, the Association for the Aid of Crippled Children and the Muscular Dystrophy Associations of America. We are especially indebted to Dr. Philip Grant, Program Director for Developmental Biology of the National Science Foundation and to Mr. Lewis B. Cuyler, President of the Association for the Aid of Crippled Children for their informed and liberal kindness and generosity.

POSTSCRIPT

In order to compare the results which the foregoing report has described for N-ethylnicotinamide we made subsequent tests with N,N-diethylnicotinamide (Nikethamide, Coramin), N-methylnicotinamide and N,N-dimethylnicotinamide. These tests were done by injecting the compounds into small groups of White

Leghorn eggs after 96 hours of incubation. The results were as follows: N,N-diethylnicotinamide was much less toxic than N-ethylnicotinamide. At a dosage of 10 mg/egg toxicity and teratogenic response were very slight. Following treatment with 20 mg/egg (an amount which with N-ethylnicotinamide quickly kills nearly all embryos), N,N-diethylnicotinamide was more toxic than the injection of half the amount of N-ethylnicotinamide; it led to malformations similar to those produced by 10 mg/egg N-ethylnicotinamide, but the incidence was lower. N-methylnicotinamide was more toxic than the N-ethyl compound, but survivors of treatment with either 5 or 10 mg/egg showed none of the tibia-fibula defects produced by N-ethylnicotinamide and only a low incidence of abnormalities of the beak. N,N-dimethylnicotinamide in dosages up to 10 mg/egg was nearly non-toxic and did not induce any malformations.

LITERATURE CITED

Bell, J. 1928 Blue sclerotics and fragility of bones. Treas. Hum. Inh., *2:* 269–324.

Boyd, E. M., and J. D. Forde 1940 Nikethamide (Coramine) and water balance. J. Pharmacol. Exp. Therap., *70:* 279–282.

Clouston, H. R. 1929 A hereditary ectodermal dystrophy. Canad. Med. Assoc. J., *21:* 18–31.

——— 1939 The major forms of hereditary ectodermal dystrophy. Canad. Med. Assoc. J., *40:* 1–7.

Dagg, C. P. 1964 Inheritance of interstrain differences in frequency of polydactyly produced by 5-fluorouracil in mice. Abstracts, Fourth Annual Meeting, Teratology Society, p. 3.

——— 1965 Experimental modification of gene penetrance. Abstracts, Fifth Annual Meeting, Teratology Society, p. 8.

Dagg, C. P., G. Schlager and A. Doerr 1966 Polygenic control of the teratogenicity of 5-fluorouracil in mice. Genetics, *53:* 1101–1117.

Davenport, H. W. 1962 Carbonic anhydrase inhibition and physiological function. Ciba Foundation Symposium on Enzymes and Drug Action ed. J. L. Mongar and A. V. S. de Reuck. London (J. and A. Churchill), pp. 16–27.

Dulce, H.-J. P. Siegmund, F. Körber and E. Schütte 1960 Zur Biochemie der Knochenauflösung. III. Über das Vorkommen von Carboanhydrase im Knochen. Zeitschr. Physiol. Chem., *320:* 163–167.

Ellison, A. C. 1965 Determination of carbonic anhydrase in the epiphysis of endochondral bone. Proc. Soc. Exp. Biol. Med., *120:* 415–418.

Hampé, A. 1959 Contribution à l'étude du développement et de la régulation des déficiences et des excédents dans la patte de l'embryon de Poulet. Arch. Anat. Micros. Morph. Exp., *48:* 345–478.

——— 1960 La compétition entre les éléments osseux du zeugopode de Poulet. J. Embryol. Exp. Morph., *8:* 241–245.

Hay, M. F., C. E. Adams and C. Lutwak-Mann 1960 The effect of certain agents upon the early rabbit embryo. Abstracts. J. Endocrinol., *20:* II–III.

Jukes, T. H., and H. P. Broquist 1963 Sulfonamides and folic acid antagonists. Metabolic Inhibitors ed. R. M. Hochster and J. H. Quastel, New York and London (Academic Press), *1:* 481–534.

Kalter, H., and J. Warkany 1957 Congenital malformations in inbred strains of mice induced by riboflavin-deficient, galactoflavin-containing diets. J. Exp. Zool., *136:* 531–553.

Kaplan, N. O., A. Goldin, S. R. Humphreys and F. E. Stolzenbach 1957 Pyridine precursors of mouse liver diphosphopyridine nucleotide. J. Biol. Chem., *226:* 365–371.

Landauer, W. 1948 The effect of nicotinamide and α-ketoglutaric acid on the teratogenic action of insulin. J. Exp. Zool., *109:* 283–290.

——— 1949 Le problème d'électivité dans les expériences de la tératogenèse biochimique. Arch. Anat. Micr. Morph. Exper., *38:* 184–189.

——— 1954 On the chemical production of developmental abnormalities and phenocopies in chicken embryos. J. Cell. and Comp. Physiol., *43,* Suppl. 1: 261–305.

——— 1956 Rudimentation and duplication of the radius in the duplicate mutant form of fowl. J. Genetics, *54:* 199–218.

——— 1957 Niacin antagonists and chick development. J. Exp. Zool., *136:* 509–530.

——— 1965 Gene and phenocopy: selection experiments and tests with 6-aminonicotinamide. J. Exp. Zool., *160:* 345–354.

Landauer, W., and E. M. Clark 1962 The interaction in teratogenic activity of the two niacin analogs 3-acetylpyridine and 6-aminonicotinamide. J. Exp. Zool., *151:* 253–258.

——— 1964a On the teratogenic interaction of sulfanilamide and 3-acetylpyridine in chick development. J. Exp. Zool., *156:* 313–322.

——— 1964b Teratogenic risks of drug synergism. Nature, *203:* 527–528.

Layton, W. M., Jr., and D. W. Hallesy 1965 Deformity of forelimb in rats: association with high doses of acetazolamide. Science, *149:* 306–308.

Lutwak-Mann, C. 1955 Carbonic anhydrase in the female reprodutcive tract. Occurrence, distribution and hormonal dependence. J. Endocrinol., *13:* 26–38.

Maren, T. H., E. Mayer and B. C. Wadsworth 1954 Carbonic anhydrase inhibition. I. The pharmacology of Diamox, 2-acetylamido-1,3,4-thiadiazole-5-sulfonamide. Bull. Johns Hopkins Hosp., *95:* 199–243.

McNutt, W. 1954 Gene expression as influenced by genetic background as illustrated by ventral tubercles on cervical vertebrae of mice. J. Hered., *45:* 235–240.

Ottley, C. M. 1932 Osteopsathyrosis (Lobstein's disease), a critical review. Arch. Dis. Child., *7:* 137–148.

Ritala, A. M. 1933 Zur Berechnung des statistischen mittleren Fehlers (standard error). Acta Societas Medicorum Fennicae "Duodecim" Ser. b, 19.

Shepard, Th. H. 1962 Carbonic anhydrase activity in early developing chick embryos. J. Embryol. Exp. Morphol., *10:* 191–201.

Siegmund, P., and H. J. Dulce 1960 Zur Biochemie der Knochenauflösung. I. Einfluss des Carboanhydratase-Inhibitors 2-acetamino-1,3,4-thiodiazolsulfonamid-(5) (Diamox) auf den Calciumstoffwechsel von Legehennen. Zeitschr. Physiol. Chem., *320:* 149–159.

Wilson, J. G., Th. H. Maren and K. Takano 1966 Teratogenicity of carbonic anhydrase inhibitors in the rat. Abstracts, Sixth Annual Meeting, Teratology Society, pp. 30–31.

Wolff, E., and A. Hampé 1959 Sur la régulation de la patte du Poulet après résection d'un segment intermédiaire du bourgeon de patte. C. R. Soc. Biol., *148:* 154–156.

Wolff, E., and M. Kieny 1962 Mise en évidence par l'irradiation aux rayons X d'un phénomène de compétition entre les ébauches du tibia et du péroné chez l'embryon de poulet. Develop. Biol., *4:* 197–213.

Zwilling, E. 1949 Reversal of insulin-induced hypoglycemia in chick embryos by nicotinamide and α-ketaglutaric acid. Proc. Soc. Exp. Biol. Med., *71:* 609–612.

Zwilling, E., and J. T. DeBell 1950 Micromelia and growth retardation as independent effects of sulfanilamide in chick embryos. J. Exp. Zool., *115:* 59–81.