BIOCHEMICAL BASIS FOR A DIFFERENCE IN RESPONSE TO A TERATOGEN
BIOCHEMICAL BASIS FOR A GENETICALLY DETERMINED
DIFFERENCE IN RESPONSE
TO THE TERATOGENIC EFFECTS OF 5-AMINONICOTINAMIDE

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I. INTRODUCTION

The application of precisely timed, short exposures of embryos to specific metabolic inhibitors is a useful method for analysing the biochemical aspects of development. The teratologic approach to the study of the biochemical requirements of various organogenetic processes in the developing embryo was first formulated by Warkany (1941) who showed that the offspring of female rats maintained on a vitamin A deficient diet had characteristic patterns of malformations. By adding vitamin A to the diet at various stages of pregnancy it was possible to modify the types of malformations produced. Thus, when vitamin A was added to the diet before the twelfth day of gestation, malformations of the aortic arch were prevented, but cardiac defects were not prevented when the vitamin was added on the tenth day. This method demonstrated that some processes of development required more vitamin than others, since some organs develop normally in spite of the vitamin deficiency, and that the vitamin A requirements of the embryo vary from one developmental stage to another.
This approach has been refined by the advent of potent and specific antimetabolites for a number of biologically important compounds. The nicotinamide antagonist 6-aminonicotinamide (6-AN) is teratogenic in mice even when a corrective dose of nicotinamide is given as little as one hour after injection of the analog. By varying the amount of 6-AN and nicotinamide supplement administered, it is possible to establish the requirements of various developmental processes for nicotinamide. For instance, when a standard dose of 6-AN is given to a pregnant mouse on day $13\frac{1}{2}$ of gestation and a standard dose of nicotinamide is given simultaneously, no malformations occur in the embryos. When the same dose of nicotinamide is given two hours after the analog almost all the embryos have cleft palate. When twice the standard amount of nicotinamide is given two hours after the analog, the cleft palate frequency is reduced, suggesting that a single dose of nicotinamide was not sufficient to correct the metabolic deficiency produced by the analog. At other developmental stages, and for other malformations, a single dose of nicotinamide might be enough to overcome the inactivation caused by the analog. In this method, we have a quantitative way to study the nicotinamide
requirements of the embryo from stage to stage of development.

In studying the effects of an environmental teratogen on development, it is important to take the genetic constitution of the reacting embryo into account, because it determines the susceptibility of the embryo to the environmental agent. In a number of instances it has been shown that the mother's genotype, as well as the embryo's, may influence the embryo's susceptibility (Kalter, 1954; Goldstein et al., 1963). Much can be learned about normal and abnormal development by investigating the interaction of an environmental factor with a variety of genotypes. A comparison of strains that react differently to a teratogen can be useful in clarifying the interaction of factors that determine whether a given embryo is malformed. For example, the A/Jax and C57BL strains differ in their susceptibility to the teratogenic effects of cortisone. In the C57BL strain, which is resistant to the action of cortisone, palate closure begins as much as 10 to 12 hours earlier than in the A/Jax strain (Walker and Fraser, 1956). If the normal time of palate closure in these strains and a variety of crosses are compared, a correspondence between the normal time of palate closure and the frequency of cortisone-
induced cleft palate becomes apparent — the lower the frequency of cortisone-induced cleft palate, the earlier the time of palate closure (Trasler and Fraser, 1958). This relationship also holds for 6-AN-induced cleft palate. It might be thought, then, that the strain difference in cleft palate frequency could be due to the fact that the teratogen has longer to act in the strain where palate closure occurs later. However, this does not seem to be the correct explanation, since the frequency of cleft palate is not increased in the C57BL strain by beginning cortisone treatment earlier (Kalter, 1954).

It would seem that the "intrinsic shelf force" which enables the shelves to push their way over the tongue and become horizontal builds up more effectively in the C57BL strain than in the A/Jax strain, and that this is reflected in an earlier time of palate closure and greater resistance to the effects of cortisone and 6-AN (Fraser, 1960). The force which moves the shelves to the horizontal position may be related to the build up of acid mucopolysaccharide in them (Walker and Fraser, 1957; Larsson, 1962).

Strain differences in response to teratogens can be useful in identifying the mode of action of the teratogens. For instance, the frequency of cleft palate
produced by cortisone or 6-AN is higher in the A/Jax strain than in the C57BL strain. On the other hand, galactoflavin produces more cleft palates in the C57BL strain than in the A/Jax strain, thus showing that the susceptibility of the A/Jax palate to cortisone and 6-AN is not due to a nonspecific instability to any environmental insult. It also suggests that galactoflavin blocks the process of palate closure at a different metabolic point than 6-AN and cortisone, which may act on the same metabolic pathway.

The interactions of genotype, environment, and teratogen can be extremely complex and the frequency of induced malformations may vary widely from strain to strain. Even environmental factors such as maternal weight and diet can influence the effect of a teratogen -- for example, on one diet a given dose of cortisone produced more cleft palates in the A/Jax than the C57BL strain, but on another diet the reverse was true (Warburton et al., 1962).

Strain differences in response to teratogens are practically all found to be multigenic when subjected to analysis (Kalter, 1954; Schlager and Dagg, 1965). None as yet been related to a specific biochemical difference. This thesis attempts to show that the difference between the A/Jax and C57BL strains
in the frequency of 6-AN-induced cleft palate is related to a difference in the efficiency with which excess nicotinamide is utilized in these strains.
II. LITERATURE REVIEW

A. Antimetabolites:

The study of biological antagonism between structurally similar substances has been intensively pursued for the past twenty-five years. In most cases one of the pair of antagonists is a chemical compound essential to the existence of the organism. Such substances, because they have important roles in metabolic reactions, are called "metabolites." They may be vitamins, hormones, amino acids, or other compounds which occur in nature and are necessary for the life process.

The structural analogs of these metabolites are most often synthetic compounds produced in the laboratory, but occasionally they occur naturally. They are of interest because some of them possess the ability to elicit the symptoms associated with a deficiency of the metabolite they resemble. As a result, these antagonistic structural analogs have been called "antimetabolites," although a more precise name would be "structural analogs antagonistic to metabolites."

It should be noted that not all analogs of a metabolite are antimetabolites of it. An analog is
merely a compound which bears a resemblance to a metabolite. An antimetabolite has, in addition to this structural resemblance, biological properties which relate its action to that of the metabolite.

There are numerous examples of antimetabolites which can produce signs of deficiency of the related metabolite in bacteria, fungi, invertebrates, and higher animals. They may also affect the participation of the metabolite in chemical reactions in cell-free systems (Woolley, 1952). However, not all antimetabolites will affect an array of organisms. Many examples are known in which an analog exerts its action only on certain species or strains of living things.

Frequently, when a structural analog of a metabolite antagonizes the action of that metabolite, it is because the two substances are competing with each other. However, antagonism can be manifested without any evidence of competition. In the former case, the effect of the antimetabolite is inversely proportional to the amount of the metabolite present over a wide range of concentration. In the latter, either the metabolite is completely unable to counteract the biological effects of the analog, or it is able to do so only over a limited range.
of concentration.

The measurement of antagonism between metabolite and antimetabolite is expressed by the inhibition index. This is the ratio of the concentration of the two substances at which their effects are just balanced. It represents the amount of antimetabolite needed to overcome a specific biological effect of a unit weight of metabolite. The inhibition index may be calculated accurately, in organisms which do not synthesize the metabolite, by determining the amounts of metabolite and antimetabolite added to the system (Woolley, 1952). In the case of organisms which can make the metabolite an inaccuracy arises from the fact that the concentration of functioning substance is the amount added to the system plus an unknown amount formed within it. (A correction for this unknown can be made by subtracting the quantity of antimetabolite needed for an effect when no metabolite is added).

The inhibition index is constant if the antagonism is competitive, since the ratio between the contending pair of substances is constant. However, if the index is not constant, but changes as the concentration of metabolite is changed, the
antagonism is non-competitive. In such cases an inhibition index is of little value.

The most plausible explanation of the phenomenon of antagonism between structurally similar compounds is that the antimetabolite is able to form a complex with an enzyme or other protein with which the metabolite usually reacts, but that the enzyme-antimetabolite complex cannot then go on to yield enzyme and normal transformation products of the substrate like the enzyme-metabolite complex. The result is that the two-stage process by means of which fresh enzyme is continually liberated is stopped, and the supply of products from the reaction is cut off.

The formation of the enzyme-metabolite complex is a reversible reaction, as is the formation of the enzyme-antimetabolite complex. In a mixture of enzyme, metabolite, and antimetabolite there are, therefore, two competing reactions. The predominating reaction will depend on: (1) the relative affinities of the metabolite and antimetabolite for the protein, and (2) their relative concentrations. If the concentration of the antimetabolite is raised in a system in which
the enzyme-metabolite complex predominates because of high relative concentration of the metabolite, a point will be reached eventually at which the metabolite will be pushed off the protein and replaced by the antimetabolite. If now the relative concentration of the metabolite is increased, the antimetabolite will be displaced and the enzyme-metabolite complex will again predominate. When this occurs in a living organism it can be said that the effect of the antimetabolite has been reversed, or stopped, and that the metabolite has been used to "protect" the organism from the effects of the antimetabolite (Woolley, 1952).

According to the favored hypothesis to explain the mode of action of antimetabolites, the combination of inhibitory agents with an enzyme, or other specific protein with which the metabolite normally reacts, is essential. If in one species this enzyme is able to form such a complex with a given antimetabolite, while in another species such a union is poorly formed, if at all, then the first organism might be susceptible, and the second resistant, to the analog. Consider, for
example, the system of enzymes which forms nicotinamide adenine dinucleotide (NAD) from nicotinamide. If in some living things the specific protein to which nicotinamide is substrate is capable of combining with the antimetabolite 3-acetyl pyridine (3-AP), then this analog should antagonize the action of the vitamin in those species. If in some other species the protein which performs the same function is different enough from that of the first class so that no combination is formed, then the analog would not harm the second class of species.

From the above discussion one can conclude that the inhibition index is a measure of the relative affinities of metabolite and antimetabolite for the particular enzymes (or proteins) with which the metabolite normally reacts. This is true for simple cell-free enzyme systems, but other factors must be considered in the case of living organisms (Hicks, 1955). When chemical agents and drugs are introduced into an animal, a number of poorly understood factors come into play to alter its even distribution and duration of action in all cells: cell membranes may differ in their permeability to the same compounds; a compound may
be detoxified at different rates in different tissues; the pathological effects of an inhibitor may be vitiated because a cell has a relatively large amount of the metabolite, or alternate metabolic pathways may offer an escape for the cell; the blood supply of one region may deliver and clear the compound more rapidly than that of another, thus modifying the duration of action even though other factors might be equal. As a result, the inhibition index as found in a living organism may represent the outcome of several factors rather than the relative affinity of the two compounds for a specific protein. The variation of the inhibition index from species to species for the same metabolite-antimetabolite pair becomes understandable in view of this, and the fact that, for a given substrate, the affinity often differs for enzymes derived from various species (Woolley, 1952).

Finally, even when two substances have a similar biochemical action in vitro it cannot always be predicted that they will be handled the same way in vivo, granted that they are both delivered to the cell. 3-AP and 6-AN, both analogs of nicotinamide (figure 1), produce different
Fig. 1

NICOTINIC ACID (NIACIN)

NICOTINAMIDE

3-ACETYL PYRIDINE

6-AMINONICOTINAMIDE
patterns of lesions in the living animal, and yet they show biochemical resemblances in respect to being incorporated into pyridine nucleotides, and their pathological effects are prevented by nicotinamide (Hicks and Coy, 1958).

B. Discovery of nicotinic acid (niacin): Like most other B vitamins, nicotinic acid (or a derivative) is required by all living cells. In animal tissues almost all the nicotinic acid is found in the nicotinamide moiety of two coenzymes which catalyze chemical reactions essential to cellular life. So far as is known, nicotinic acid has no metabolic function other than that exerted through the coenzymes of which it is a part.

Specific biochemical functions for nicotinic acid had been discovered before it was known that this substance was a vitamin. In 1934 Warburg and Christian found that nicotinamide was part of the coenzyme II molecule. In 1936 they isolated it from coenzyme I. It was not until 1937, however, that the true nutritional and biological significance of nicotinic acid became evident following the reports of Elvehjem et al. (quickly confirmed by Street and Cowgill, 1937) that pure
Nicotinic acid would cure experimentally-produced blacktongue in dogs, and that nicotinamide could be isolated from liver concentrates (Elvehjem et al., 1938) which had been shown to be active in curing blacktongue by Koehn and Elvehjem (1936). These observations suggested that nicotinic acid might be useful in the treatment of pellagra, since canine blacktongue and human pellagra were thought to be analogous or closely related diseases — all food substances curative or preventive for one were for the other, too. Late in 1937 Fouts et al., Smith et al., and Spies et al. almost simultaneously announced that nicotinic acid would cure pellagra, a fact confirmed by numerous reports since then. The almost universal biological significance of nicotinic acid was established by these studies and others, such as those of Knight (1937), Mueller (1937), and Landy (1938), showing that it was an essential growth factor for a variety of bacteria and other organisms.

C. Nicotinic acid-containing coenzymes:

Since these early studies numerous biochemical reactions have been found to depend on nicotinic acid-containing coenzymes. So far, there are still only
two coenzymes known to contain nicotinic acid: coenzyme I and coenzyme II, which are described under various names in the literature. Coenzyme I is most often called diphosphopyridine nucleotide (DPN) or nicotinamide adenine dinucleotide (NAD); while coenzyme II is usually referred to as triphosphopyridine nucleotide (TPN) or nicotinamide adenine dinucleotide phosphate (NADP).

NAD and NADP take part in oxidation-reduction systems by virtue of their ability to accept hydrogen atoms (dehydrogenation) from certain substrates and transfer these hydrogen atoms to other hydrogen acceptors, such as the flavin enzymes. They function by reversibly alternating between the oxidized and reduced states.

Before they can participate in biochemical reactions NAD and NADP must be joined with, or activated by, specific proteins (apodehydrogenases). Although the exact mechanism by which the protein apoenzyme unites with the coenzyme is unknown, it is clear that the coenzyme will not function catalytically if the apoenzyme is not present. The apoenzymes are quite specific — a different one being required for each substrate system; while the coenzymes are relatively non-specific, in that they
function in a number of systems involving hydrogen transfer. NAD and NADP are usually readily
dissociable from their apoenzymes; however, reduced NAD and NADP seem to have a lower affinity
for their apoenzymes than the oxidized forms.

D. Tissue distribution and intermediary
metabolism of nicotinic acid and nicotinamide:
The tissue distribution and intermediary
metabolism of administered nicotinic acid and
nicotinamide has not been extensively studied. Roth
and associates (1948) have applied radioactive
tracer techniques to this problem and obtained
interesting results. They injected C\textsubscript{14}-carboxyl-
labeled nicotinic acid and nicotinamide intra-
peritoneally in mice and determined the amount of
radioactivity appearing in exhaled air, urine,
faeces, and tissues as a function of time.

A single dose of 0.7 mg of nicotinic
acid, which exceeds the normal daily requirement
of mice, resulted in the excretion of a large amount
of radioactivity during the first 24 hours in both
urine (60% of the administered dose) and CO\textsubscript{2} (3% of
the administered dose). Excretion of the tracer
in the urine was much reduced after 24 hours, and
minimal after 48 hours. The decrease in the slope
of the urine curve after 24 hours was probably due to the incorporation of nicotinic acid and nicotinamide into the metabolic pool of the animal, whereas the initial rapid excretion represented unmetabolized injected material. However, the rapid initial excretion of labeled CO₂ was the result of normal metabolism or detoxification. After 48 hours, when administered nicotinic acid and nicotinamide have been incorporated into coenzymes, a comparison of the radioactivity found in CO₂ and urine indicated that 15-20% of the fixed nicotinic acid and nicotinamide was eliminated as CO₂. Fecal excretion of radioactive material, if there was any, was very low.

The uptake of injected radioactive nicotinic acid or nicotinamide by various tissues and organs was determined, and the concentration followed at intervals for 15 days. Uptake was highest in the kidneys and lowest in erythrocytes. No radioactivity was found in the plasma after 24 hours. The turnover rates also varied in different organs, excretion half-times being about 4 days in liver, kidney, and spleen; 5 days in cardiac muscle and erythrocytes; and 8
days in brain, sternum, and skeletal muscle.

It is worth noting that in all the experiments just described the gross metabolism of nicotinic acid and nicotinamide was demonstrated to be identical in the mouse.

Dietrich and Ahuja (1963) studied the ability of ascites tumor cells from mice to assimilate C¹⁴-labeled nicotinic acid and nicotinamide and increase their intracellular concentrations of these substances. They found that nicotinic acid entered the cell, or was adsorbed on to the cell membrane, at a much lower rate than nicotinamide, which permeated the cell membrane freely and rapidly established equilibrium between the inside and outside concentrations of free nicotinamide (regardless of temperature, pH, and other changes in the extracellular environment).

Ascites cells can apparently concentrate nicotinic acid and nicotinamide by "passive" uptake. The reaction does not appear to require metabolic energy, since the addition of deoxyglucose caused no change in the ability of the cells to concentrate these compounds. Simple adsorption on the cell membrane or an intracellular particle would explain the data except for the fact that fragmented cells
lost their ability to concentrate the vitamin. A situation must exist in which the extracellular environment is favorable to the transport of the metabolite across the cell membrane, and the internal environment is favorable for trapping it. This type of nonionic diffusion is found in kidney metabolism.

Labeled nicotinamide appeared in the coenzyme fraction within 6 minutes after it was added to the cell cultures. Since under these conditions the NAD concentration of these cell types remained unchanged, it was assumed that the incorporation of labeled nicotinamide into NAD within an intact cell was brought about by an exchange reaction between free and bound nicotinamide mediated by the enzyme, NADase.

E. Nicotinic acid analogs:

1. 3-acetylpypridine (3-AP)

Soon after it was demonstrated that nicotinic acid (NA) and nicotinamide (N) were active in the prevention and cure of blacktongue in dogs, studies were undertaken by Woolley et al. (1938) to determine the anti-blacktongue potency of various compounds related to nicotinic acid. The results indicated that a highly specific
structure was required for anti-blacktongue activity. It appeared likely that only those compounds which were capable of oxidative or hydrolytic conversion to nicotinic acid and/or nicotinamide possessed anti-blacktongue potency. Woolley and his associates noted that two compounds, pyridine-3-sulfonic acid (P-3-SA) and 3-acetylpyridine (3-AP), not only failed to alleviate blacktongue in dogs on deficient diets, but killed them at doses which were not harmful to normal animals. They were unable to explain this observation at the time and suggested that these compounds were probably inactive.

It was not until 1945 that Woolley investigated the anti-vitamin activity of P-3-SA and 3-AP. P-3-SA was found to be inactive in mice, but 3-AP was observed to cause a disease characterized by many of the symptoms seen in nicotinic acid-deficient dogs and humans. Since sufficient quantities of nicotinic acid or nicotinamide suppressed the appearance of the disease, it was concluded that 3-AP is an anti-metabolite which can cause nicotinic acid deficiency in animals --- even those normally requiring no exogenous source of nicotinamide, such as mice and
rats. The following year Woolley (1946) showed that tryptophan, a precursor of nicotinic acid, was as active as nicotinic acid in counteracting the biological effects of 3-AP in mice.

Gaebler and Beber (1951) reported marked increases in urinary excretion of N¹-methyl-nicotinamide (N¹MeN), a normal end-product of nicotinic acid metabolism, in both normal and niacin-deficient dogs fed 3-AP. This could be interpreted as evidence that 3-AP, acting as an antivitamin, displaces nicotinamide from its nucleotides, or prevents its incorporation therein. Alternatively, 3-AP might be biologically oxidized to nicotinic acid, methylated, and excreted, in which case it would be acting as a provitamin. Beher et al. (1952) tested these possibilities by feeding labeled 3-AP (C¹³ in carbonyl group) to dogs and rats. If N¹MeN excreted after labeled 3-AP arises only from displaced nicotinamide or unutilized nicotinic acid formed from tryptophan, it should not be labeled; however, if it is derived from nicotinic acid arising by direct oxidation of 3-AP it should be labeled. Their experiments indicated that 3-AP is oxidized to nicotinic acid to an extent sufficient to account
for the increased N\textsubscript{1}MeN level observed following its ingestion.

Beher and Anthony (1953) reported that between 20 and 30 per cent of injected 3-AP could be detoxified to nicotinic acid and Beher et al. (1953) found that canine blood cells, and the kidney were sites of detoxification, but the liver was responsible for the major part of 3-AP conversion. The latter is interesting in view of the fact that coenzyme I and II contents of this organ are adversely affected by nicotinic acid deficiency, and these enzymes are involved in the oxidation of 3-AP to nicotinic acid. Therefore, one would expect nicotinic acid-depleted animals to metabolize 3-AP at a retarded rate. They were found to tolerate only a fraction of the 3-AP normal dogs can handle, as Woolley et al. reported in 1938. The difference in toxicity is probably related to the detoxification rate (influenced by coenzyme concentration) as well as to anti-vitamin activity -- the effects being additive.

McDaniel (1953) was intrigued by the fact that 3-AP had been reported to possess both
anti-niacin activity in dogs (Woolley et al., 1938) and mice (Woolley, 1945) and niacin-like activity in rats and dogs, in that it serves as a precursor of urinary N₁MeN (Beher et al., 1952). He did experiments to determine the effects of 3-AP in the prevention and treatment of blacktongue in dogs fed niacin-deficient and control diets. Daily doses of 60 mg. of 3-AP prevented blacktongue over long periods in dogs fed niacin-deficient diets, and 25-60 mg. per day were sufficient to cure blacktongue. However, niacin-depleted dogs appeared to have a diminished capacity to convert 3-AP to nicotinic acid, as shown by urinary N₁MeN excretion studies and by the fact that doses of 25-260 mg. of 3-AP were highly toxic in deficient dogs but relatively well tolerated by normal dogs (McDaniel et al., 1955). Nicotinic acid prevented 3-AP toxicity if administered before the deficiency syndrome developed; however, once started, the toxic syndrome was irreversible, even though the analog was discontinued and the animal fed large doses of nicotinic acid. In this respect the syndrome was different from the disturbances seen in cases of niacin deficiency produced by dietary
deprivation. These data suggest that the animals studied have a limited capacity to transform 3-AP to nicotinic acid. When the dosage of 3-AP exceeds this capacity, the 3-AP molecule acts as a specific antagonist to niacin.

These anomalous, but intriguing, findings were explained by Kaplan et al., (1954). This group discovered that animal tissue NADases can catalyze an exchange reaction between the nicotinamide moiety of NAD and pyridine compounds related to nicotinamide, such as 3-AP, ethyl nicotinate, isonicotinamide, etc. The fact that analogs of NAD could be formed in vitro suggested that antimetabolites of nicotinamide might exert their pharmacologic action by such a mechanism. They were able to show that these exchanges do occur in vivo by studying the toxic action of 3-AP. They found that either nicotinamide or NAD, when given simultaneously with 3-AP, protected against its toxicity in niacin-deficient animals, whereas nicotinic acid or tryptophan did not. The fact that nicotinamide can protect against the toxicity of this antimetabolite and nicotinic acid cannot is of interest because nicotinic acid and nicotinamide
are considered to be of equal value in the diet. The inability of nicotinic acid to protect against the toxicity of 3-AP appears to be related to the finding that tissue NADases do not promote exchange between the nicotinamide of NAD and free nicotinic acid; they will only catalyze exchanges between bound nicotinamide and free nicotinamide. The ineffectiveness of nicotinic acid indicates exchange reactions do occur in vivo, since, if a synthetic system were involved, one would expect nicotinic acid to have protective activity because it can replace nicotinamide in the diet. This demonstrated for the first time how important it is in studying an antimetabolite to know whether it acts by competing with a metabolite in a synthetic reaction (that is, synthesis of NAD from nicotinamide), or in exchange reactions of the NADase type.

3-AP is unique in that it is an antimetabolite that can be detoxified by its conversion to the metabolite. Kaplan and his associates (1954) found that injection of a given amount of 3-AP in mice led to a 4-fold increase of NAD in the liver; the same amount of nicotinamide produced an 8-fold increase of NAD. However, no 3-AP
analog of NAD was found in the liver. It would appear that the liver can convert 3-AP to nicotinamide and thereby detoxify a large amount of 3-AP (this could explain the preventive and curative effects of 3-AP in niacin deficiency); however, the conversion to nicotinamide does not occur in a number of other tissues. In brain and splenic tissue there is no increase in NAD concentration after 3-AP administration and the 3-AP analog of NAD is formed. Therefore, it would appear that when the rate of detoxification of 3-AP by the liver is exceeded, it is distributed to other tissues and there incorporated into NAD analogs by way of the NADase system.

Whether the toxic effects of 3-AP are due to the analog itself, or are caused by a decrease in the tissue level of NAD resulting from the formation of the analog is not clear. Preliminary experiments in which the 3-AP analog of NAD is injected into mice indicate that it is not as toxic as free 3-AP (Kaplan et al., 1954). Since coenzymes are the functional forms of vitamins and as such occupy key positions in metabolic processes, a decrease in their effective
concentration by the formation of analogs would be expected to interfere with the normal functions of cells.

The 3-AP analog of NAD was the first pyridine-substituted analog of NAD found to be active in a number of dehydrogenase reactions (Kaplan et al., 1956). Not only did it react with some dehydrogenases, but it did it faster than NAD in a few cases (horse liver dehydrogenase, for example). It also has many properties similar to those of NAD — it can be reduced, enzymatically or chemically, with hydrosulfite; and it forms complexes (called addition reactions) with cyanide, bisulfite, and dihydroxyacetone (Kaplan and Ciotti, 1956). A comparison of the equilibrium constants of the addition reactions of NAD and its 3-AP analog is interesting. The ratio of the constants (3-AP analog to NAD) seems to vary with the different reactions.

2. 6-aminonicotinamide (6-AN)

A second compound which leads to symptoms of niacin deficiency in vertebrates — 6-aminonicotinamide (6-AN) — was reported by Johnson and McColl in 1955. It was first noticed that 6-AN was extremely toxic to rabbits and rats (Johnson,
1955), causing paralysis of the extremities even at low dosage levels. The fact that this agent had a delayed effect suggested it might be an antimetabolite of nicotinamide. This was confirmed by experiments with rats in which it proved to be a more potent antagonist of nicotinamide than 3-AP (LD$_{50}$ almost 10 times that of 3-AP).

The LD$_{50}$ of 6-AN in CF-70 mice was found to be 35mg/kg body weight. Simultaneous administration of 50mg/kg of nicotinamide resulted in an 8-fold increase in the LD$_{50}$ of 6-AN. Nicotinic acid and tryptophan were also found to give protection when administered simultaneously with 6-AN, although they were less efficient.

On the assumption that 6-AN might give rise to an inactive NAD analog, the rate of O$_2$ uptake of tissues from treated and normal animals was compared. In the absence of added substrate the O$_2$ uptake of mouse liver homogenate from treated animals was only 30 per cent that of the controls. Treated mice were depleted of both NAD and oxidizable substrate, since the addition of each, in vitro, resulted in a greatly increased rate of oxidation, while addition of both together gave a normal rate. There was no
effect on O₂ uptake if 6-AN was added in vitro to the liver homogenate of normal mice. The authors cited Kaplan's work (1954) and concluded that the toxicity of 6-AN was probably due to the formation of an inactive NAD analog with consequent depletion of NAD in some tissues.

The following year the 6-AN analog of NAD was isolated in good yield from an incubation mixture containing pig brain NADase, NAD, and 6-AN (Johnson and McColl, 1956). It was found to be inactive in the yeast alcohol dehydrogenase system, but able to undergo an addition reaction with cyanide. (The validity of the addition reaction has been questioned by Friedland et al. (1958), Dietrich et al. (1958), and Anderson (1958)). The analog was also detected by chromatographic and spectrophotometric techniques in the liver and kidneys of mice given 6-AN and in Walker 256 tumors of treated rats.

Johnson and McColl (1956) showed that 6-AN exhibits a cumulative effect in rats. Subcutaneous injections of 2 mg/kg body weight/day for 10 days resulted in 50 per cent mortality on the 11th day. Walker tumor-bearing rats appeared to withstand the lethal effects of the
antimetabolite better than did the non-tumor bearing controls.

In 1957 two groups (Halliday et al. and Shapiro et al.) demonstrated the inhibitory activity of 6-AN against experimental tumors in mice. Halliday and associates reported that 6-AN was comparatively active against 6C3HED lymphosarcoma and C3H mammary adenocarcinoma, but relatively inactive against several other tumors. 6-amino-nicotinic acid (6-ANA) was about one seventh as active as 6-AN. The tumor inhibition of both of these agents was reversed by nicotinamide. Shapiro and his group found regression of mammary adenocarcinoma 755 at drug levels of 3-4 mg/kg body weight was accompanied by a 20 per cent weight loss. Combining 6-AN treatment with injections of 8-azaguanine and testosterone reduced the weight loss without changing the degree of tumor regression.

Shapiro et al. (1957) also measured the NAD concentrations of various tissues to obtain information on the possible biochemical role of niacin antagonists. The effect of 6-AN on the concentration of NAD in mouse liver, tumor, and brain tissue was studied. Daily
administration of 30 mg/kg body weight of 6-AN for 3 days lowered the NAD level of liver slightly, but had no effect on the NAD concentration of brain and mammary tumor 755 tissue. The data demonstrate that normal NAD synthesis is not affected by 6-AN. However, another niacin antagonist, 2-ethylamino-1,3,4-thiadiazole, increased the amount of NAD in various tissues.

Friedland et al. (1958) and Dietrich et al. (1958a and b) reported that 6-AN tumor therapy in mice competitively inhibits the activities of various pyridine nucleotide-dependent enzymes, particularly those associated with mitochondrial oxidative phosphorylation, such as:β-hydroxybutyrate and α-ketoglutarate dehydrogenase. These enzymatic changes are probably not due to interference with coenzyme synthesis by 6-AN, since no significant change in the total nucleotide concentration of 755 tumor tissue is observed following treatment with 6-AN. However, there is a drastic decrease in the ATP and ADP level in this tissue, with a concomitant increase in AMP.

The 6-AN analogs of NAD and NADP were
isolated from 6-AN treated mice by Dietrich et al. (1958a). These compounds underwent none of the addition reactions typical of natural pyridines. This observation, and those mentioned above, suggest that the in vivo mechanism of action of 6-AN is one of replacing a normal coenzyme with a faulty one unable to function in oxidation-reduction reactions. The authors postulated that 6-AN exercises its inhibitory effect only after it is converted to nonphysiological NAD analogs. These unnatural pyridine nucleotides must compete with the normal ones in the cell for the apodehydrogenases. Thus, the level of NAD and NADP present in the cell is an important factor with respect to the ability of 6-AN to antagonize the NAD-dependent enzymes of that cell.

Inhibition is thought to occur at the initial step of electron transport in the mitochondria. The abnormal coenzymes may become irreversibly bound to apodehydrogenases forming a pseudocoenzyme-apoenzyme complex incapable of functioning in the normal electron and hydrogen transfer reactions. Normal dehydrogenation stops and no electrons enter
the cytochrome system. As a result, a deficiency in the respiratory capacity of the cell is produced and oxidative phosphorylation is inhibited, thereby lowering the high energy phosphate pools (Dietrich et al., 1958a).

This hypothesis concerning the inability of the 6-AN analogs of NAD and NADP to function as normal electron carriers, receives confirmation and explanation from the theoretical studies of Pullman and Pullman (1959, 1960) on the relation between the electronic structure and the functioning of respiratory coenzymes. They have shown that the oxidation-reduction mechanism of the respiratory enzymes, pyridine nucleotide enzymes, or flavoproteins, may be related to the energies of the molecular electronic orbitals of the oxidized and reduced forms of the associated coenzymes. A particularly low-lying empty orbital is associated with the oxidized form, and a particularly high-lying filled orbital is associated with the reduced form. The oxidized form thus has a great tendency to accept electrons, and the reduced form to give them up: the oxidation-reduction being accomplished
by the appropriate instantaneous redistribution of these orbitals.

The quantum-mechanical calculations of the authors show that the lowest empty molecular electronic orbital is placed much higher in the 6-AN analog of NAD than in NAD itself. Consequently, the faulty coenzyme does not possess the pronounced electron-acceptor properties of the natural coenzyme and cannot thus function as an electron carrier in the respiratory chain. The antitumor activity of the antagonist may be considered, in agreement with the hypothesis of Dietrich and associates (1958b), as resulting from this loss of electron-accepting capacity.

Previous studies have demonstrated that antimetabolites do not antagonize all systems dependent on the metabolite to the same degree (Woolley, 1952). Dietrich et al. (1958b) showed that 6-AN antagonizes various NAD-dependent enzymes in a similar manner in mice. At a therapeutic dose (2mg/kg for 8 days) the oxidation of β-hydroxybutyrate and malate to citrate and α-ketoglutarate to acetoacetate is blocked in tumor and lung
tissue, which have low NAD concentrations, but not in brain, liver, heart, and kidney tissue, which have much higher enzymatic activities and NAD concentrations. If the dose of 6-AN is increased, an increasing toxicity, as measured by enzyme inhibition, is produced in tissues with high concentrations of NAD and high NAD-dependent enzymatic activities.

Several possible reasons why some tissues, such as skeletal muscle, form little 6-ANAD are set forth: 6-AN may not be able to cross some cell membranes efficiently; cells may not possess the ability to synthesize 6-AN nucleotides from 6-AN to the same extent; some tissues may have a slow turnover of nucleotides; or cells may differ in the extent to which they can catalyze exchange reactions between 6-AN and NAD. Any one of these reasons may explain why no enzymatic antagonism is observed in these tissues after 6-AN administration.

The capacity of a tissue to utilize an antagonist and the concentration of the normal metabolite in the cell are important factors in enzyme inhibition. However, lung tissue, with an NAD:6-ANAD ratio similar to
brain, liver, and kidney, is as sensitive enzymatically to 6-AN as tumor tissue. Dietrich et al. (1958b), therefore, conclude that if 6-AN is utilized by a tissue to a degree sufficient to produce an effective coenzyme: antagonist ratio, the primary factor affecting enzyme antagonism would appear to be the enzymatic capacities of the tissue.

The same group synthesized the 6-AN analogs of NAD and NADP in vitro in 1958 and found, contrary to the report of Johnson and McColl (1956), that these analogs undergo none of the addition reactions which occur with normal pyridine nucleotides. They concluded that the presence of an amino group at the 6-position of the nicotinamide molecule either prevents the normal addition reactions at position 4, or inhibits the spectral changes expected to occur when such reactions take place.

To date there is little information available on the effect of 6-ANAD on enzymatic processes. Coper and Neubert (1964a) have prepared 6-ANAD in good yield and are studying the interference of this analog with enzymes
connected with ATP synthesis, since the toxic symptoms produced when 6-AN is administered to experimental animals suggest a lesion of a major energy-generating system is involved.

Working with the NAD-phosphorylating system reported by Griffiths and Chaplain (1962), they have found that sheep-heart mitochondria convert appreciable amounts of NAD to its phosphorylated form (NADH P) in the presence of succinate. Spectrophotometric characteristics of the compound formed led to the assumption that phosphorolysis at the 6-position of the nicotinamide ring might be involved.

When 6-ANAD is added to the reaction mixture plus NAD, formation of the phosphorylated compound is reduced. 3-APAD also inhibited formation of NADH P (to about 30-50 per cent); however, free nicotinamide derivatives (3-AP, 4-AP, and 6-AN), as well as, 4-APAD were ineffective under the conditions of the experiment.

Coper and Neubert (1964a) tested the possibility that the transphosphorylation reaction leading from NADH P to ATP might be affected equally well. Experiments were carried
out with submitochondrial particles whose respiratory chain had been blocked by antimycin A. High concentrations of 6-ANAD failed to elicit a significant decrease in ATP formation when added to the incubation mixture. Therefore, it seems probable that this analog in some way interferes with reactions leading to the formation or release of NADH P from the mitochondria, but not with the transphosphorylating reaction. Further study of the effect of 6-ANAD and 3-APAD on oxidative phosphorylation might provide information as to whether NADH P participates in oxidative phosphorylation reactions, since it need not necessarily lie in the main route of the phosphorylative sequence.

F. Teratogenicity of nicotinamide analogs:

1. Experiments on chick embryos

Only two compounds have been identified and studied which produce symptoms of nicotinamide deficiency in higher animals. They are 3-AP and 6-AN.

Woolley (1945) reported that typical symptoms of niacin deficiency appear in mice following the administration of 3-AP. These effects could be forestalled by giving either
nicotinic acid, nicotinamide, or tryptophan (Woolley, 1946). Ackermann and Taylor (1948) studied the effects of 3-AP on developing chick embryos by injecting the compound into the yolk sac of eggs that had been incubated for 96 hours. The resulting embryos showed "undersized deformed legs, and a general edematous-like condition over the surface of the body." These abnormalities could be prevented by the simultaneous administration of nicotinamide or, to a lesser extent, nicotinic acid and tryptophan. Additional experiments with 3-AP were reported by Zwilling and DeBell (1950). They found that injecting 3-AP into the yolk sac of 5-day chicks produced shortened upper beaks, general growth retardation, and thinning of the long bones. They also confirmed Landauer's observation (1948) that above an optimal level nicotinamide becomes teratogenic and induces some of the symptoms which, at lower doses, it prevents.

Nicotinamide was found in subsequent studies to possess the ability to forestall the teratological consequences in developing chick embryos of a number of compounds such as: insulin (Landauer, 1948; Landauer and Rhodes,
1952), eserine sulfate (Landauer, 1949), sulfanilamide (Zwilling and DeBell, 1950), pilocarpine hydrochloride (Landauer, 1953, 1956), and 6-AN (Landauer, 1957). The heterogeneous chemical nature of these teratogens, as well as the morphological differences between the defects they elicit, make it unlikely that these compounds act by causing identical biochemical damage, despite the fact that nicotinamide protects against the effects of all of them. However, these observations did indicate that nicotinamide played an important role in many steps of morphogenesis in the embryo, presumably as part of the pyridine nucleotides. Hence, it was of interest to study the effects on development of compounds known to be nicotinamide competitors. Landauer began working with 6-AN and 3-AP in 1957. His experiments showed that both these agents interfered with the development of 4-day chick embryos, and that supplementary nicotinamide counteracted the toxicity and prevented the developmental aberrations which followed treatment. There was little doubt, therefore, that both compounds interfered with the synthesis
and/or utilization of nicotinamide by early chick embryos. However, there were striking dissimilarities in the system specificities and teratogenic effects of each compound. Treatment with 6-AN produced chiefly skeletal malformations such as micromelia, parrot beak, and general reduction of body size (confirmed by Murphy et al., 1957; Dagg and Karnofsky, 1958; Murphy, 1960), whereas, the major effect of 3-AP was muscular hypoplasia, although there was some thinning of the long bones and shortening of the upper beak. The incidence and severity of the abnormalities was dependent on the dose for both analogs.

In seeking information on the nature of the system-specific responses to the two analogs, Landauer (1957) tried to determine if certain metabolites, other than nicotinamide, given in combination with competitors are effective in reducing toxicity and preventing abnormal development. In the case of embryos treated with 6-AN he found that L-tryptophan hydrochloride or 3-hydroxy-anthranilic acid sodium, were effective in reducing the toxicity and teratogenicity of the antimetabolite when
given as supplements; however, 10 to 20 parts of L-tryptophan must be used to replace one part of nicotinamide. Since it was known that the unincubated egg contained only traces of nicotinic acid and that synthesis of the vitamin commenced in the early stages of development (Snell and Quarles, 1941; Levy and Young, 1948), he concluded -- incorrectly, as we now know -- that the pathway from tryptophan and anthranilic acid to nicotinic acid and its amide was blocked by 6-AN. When 5-methyl-DL tryptophan hydrochloride, an effective antimetabolite of tryptophan, was injected into 4-day chicks, it was found to have effects very similar to those of 6-AN (severe micromelia, parrot beak, etc.).

Supplements of tryptophan or anthranilic acid were also beneficial after 3-AP treatment. The toxicity and teratogenicity of the antimetabolite were both lessened; however, the degree of protection afforded was far below that found for 6-AN.

The most interesting results of this study are concerned with the dissimilar systemic effects which 6-AN and 3-AP have on developing chick embryos. Even if it is postulated that
the two antimetabolites inhibit different biochemical reactions of niacin in a more or less selective way, it is difficult to explain the origin or nature of the differential system-specific responses of the chick embryo. While discussing the fact that 3-AP produces signs of nicotinic acid deficiency in mice and dogs but not in microorganisms, Woolley (1950) said, "to explain its selectivity of action, we must conclude either that the animals have functions for nicotinic acid which the microorganisms lack and with which β-acetyl-pyridine interferes, or that the specific proteins with which nicotinic acid reacts in animals differ significantly from similarly reactive proteins in bacteria so that the analog will no longer combine with them." The latter argument, which may be applied as well to different organs and systems within one organism, has found strong factual support in the work of Kaplan and associates with 3-AP (1956). It may also be applied to the reactions of two different but related antimetabolites. The existence of minute differences in the response to teratogenic stresses of various primordia has been shown by Fell (1956).
Bone explants from a 6-day chick embryo cultured on medium containing a high concentration of vitamin A were observed to be growing slower than controls after two days. It was interesting that the different rudiments did not all respond to the presence of excess vitamin to the same extent. The effect was greatest in the femur and tibia, slightly less severe in the humerus and least in the radius and ulna. This phenomenon presumably reflects the divergence of metabolic patterns which can occur in cells of identical genotype (Markert, 1956).

In 1962 Landauer and Clark returned to the question of the nature of the system-specific responses to 6-AN and 3-AP. They gave simultaneous injections of both compounds to determine if they interact or interfere with one another. Above a minimum level the pattern of anomalies typical of 6-AN, leg and beak defects, was prevented by simultaneous treatment with 3-AP. The converse was not the case, however, in that 6-AN did not interfere with, but exacerbated to quite a degree, the teratogenic effect of 3-AP.

In vitro tests by Kaplan and Giotti
(1956) and Kaplan et al. (1956) have shown that 3-AP is substituted for nicotinamide in NAD and that such 3-APAD is at least partially active in enzyme systems in which NAD functions metabolically. Landauer and Clark feel that the effect of 3-AP on the teratogenicity of 6-AN demonstrated in this study may be in vivo evidence for Kaplan's conclusion.

Another possible explanation for the protective effect of 3-AP on 6-AN, when they are administered simultaneously, arises from the work of Gaebler and Beher (1951), Beher et al. (1952), McDaniel (1952), Beher and Anthony (1953), and especially Kaplan et al. (1954). They showed that some of the 3-AP administered to animals was converted in vivo to nicotinamide. Kaplan et al. demonstrated that this conversion occurs in the liver of mice. If the yolk sac membrane of chick embryos can perform the same function, the suppressive action of 3-AP on 6-AN teratogenicity could be explained.

Johnson and McColl (1956) and Dietrich et al. (1958) reported that 6-AN can replace nicotinamide in NAD, in vitro as well as in vivo; however, this 6-ANAD is metabolically
inert. Therefore, when both analogs are injected into a chick one would expect the presence of 6-AN to compete sufficiently in the formation of NAD to result in potentiation, in incidence and degree, of the malformations produced by 3-AP, as was found to be the case (Landauer and Clark, 1962). However, increasing the amount of 6-AN above a certain level resulted in the formation of more 6-ANAD than 3-APAD and an apparent lowering of the teratogenic effectiveness of 3-AP. What really happened was that there was less 3-AP-induced damage, because less 3-APAD was formed, but an increase in 6-AN-caused malformations.

An earlier discussion of the teratogenic role these two niacin analogs play in chick development left open the question of whether affected parts have specific affinities for a particular substituted coenzyme, or whether the analogs are distributed uniformly but call forth differential responses (Landauer, 1957). The present study showed that each of the analogs reached all those parts of the embryo which respond to either of them. However, it is possible that the enzymes most inhibited
in their function by either of the two analog-substituted pyridine nucleotides occur in different amounts in embryonic parts which show teratogenic reactions.

The observations of Kaplan et al. (1956) and Dietrich et al. (1958) indicated that 3-AP- and 6-AN-substituted pyridine nucleotides of NAD differ in their enzymatic affinities. Landauer and Clark feel that the sources of their teratogenic specificities lie in this direction.

Landauer and Clark (1964) investigated again the remarkable interaction of 6-AN and 3-AP, this time to determine if it was due to the fact that both teratogens were analogs of nicotinamide. They wondered if two chemically unrelated compounds with comparable teratogenic activities would produce similar results. They used 3-AP in combination with sulfanilamide, which has effects similar to those of 6-AN (Ancel and Lallemand, 1942; Ancel, 1945) and which, while responding to supplements of nicotinamide, is not chemically related to it. Supplementation with 3-AP provided protection against sulfanilamide-induced micromelia and parrot beak. 3-AP was
incorporated into substituted pyridine nucleotides and probably played the same role it does in forestalling the occurrence of similar symptoms after 6-AN administration. Exaggeration of the teratological effects of 3-AP was found with sulfanilamide, as with 6-AN in the previous study.

The potentiating effect of 6-AN supplementation on the teratogenicity of 3-AP is probably brought about by the competitive attachment of the two niacin analogs to sites critical for muscular development; the chances for normal morphogenesis being reduced by the metabolically inert nature of 6-ANAD. An analogous mechanism may play a role in the synergistic effects of combined sulfanilamide and 3-AP administration. In the sites sensitive to 6-AN and sulfanilamide (primordia of long bones and mandible) 3-APAD may provide a helpful function as a substituted pyridine nucleotide; whereas, damage to sites sensitive to 3-AP (skeletal muscle and maxilla) is increased by the presence of 6-AN and sulfanilamide, which cause further injury by their participation in
metabolic activity.

The observations of this investigation indicated that the specific effects of teratogens were not brought about by selective distribution of toxic compounds, but by selective responses of particular sites to them.

In a final experiment using these compounds, Landauer and Clark (1964) administered individually harmless amounts of sulfanilamide and 6-AN, which lead to related malformations when used singly and in requisite dosages, to chick embryos and found that they produced a high frequency of micromelia and parrot beak when administered in combination.

2. Experiments on rat and mouse embryos

The first indication that a nicotinamide deficiency might be teratogenic in mammals came from a study by Pike (1951) on the effects of high and low tryptophan diets on the developing rat eye. A tryptophan-deficient diet induced cataracts in rat embryos, but it was not possible to prevent these cataracts by adding tryptophan to the diet at a level considered adequate for an adult rat. However, the same amount of tryptophan plus a quantity
of nicotinamide did prevent the cataracts. Pike concluded that the cataracts seemingly produced by a tryptophan deficiency might actually be due to the limited amount of tryptophan available for conversion to niacin, and, therefore, be caused by a niacin deficiency.

The teratogenicity of nicotinamide deficiency was not studied again in mammals until 1957. By this time it had been shown that some vitamin antagonists are teratogenic even without a dietary deficiency of the vitamin concerned. Thus the deficiency could be imposed quite simply at given stages of development. The first teratological investigation to use this technique in mammals with a nicotinamide competitor was that of Murphy et al. (1957). They studied the teratogenic effects of several compounds, among them 6-AN, on both rat and chick embryos. This comparison was of particular interest because chick embryos represent an independent and isolated system, whereas mammalian embryos are closely involved with the maternal host which may excrete, detoxify, or otherwise dispose of noxious chemicals and protect
the fetus.

6-AN produced a characteristic syndrome of skeletal abnormalities (parrot beak, micromelia, bending of the tibiotarsus, etc.) when injected into the yolk sac of 4-day chick embryos. This syndrome was forestalled by simultaneous administration of nicotinamide, as is the case with similar syndromes produced by a variety of other agents.

In rats there was little difference between the dose of 6-AN toxic to the mother and that which harmed the fetus; doses which caused consistent injury to the embryo were also lethal or debilitating to the mother. The LD$_{50}$ of 6-AN was in the range of 8-15 mg/kg body weight depending on the stage of gestation of the rat. 6-AN treatment of pregnant rats (8mg/kg) on day 10, 11, or 12 of gestation severely affected fetal development, causing stunting, defects in ossification, and malformations of various types (syndactyly, club feet, cleft palate, rib anomalies, etc.) depending on the stage of development when the antagonist was administered. Again, these effects could be prevented by prior administration of nicotinamide.
In 1959 Pinsky and Fraser reported that a single intramuscular injection of 6-AN (0.4–0.6 mg) caused growth retardation, skeletal abnormalities, and cleft palate in the offspring of pregnant mice treated on day 10 or 11 of gestation. They presented evidence that the frequency of an induced malformation varies with the dose of the drug and the weight of the mother. Thus, cleft palate frequency rose from 4% to 55% in the offspring of treated females of approximately the same weight when the dose of 6-AN was increased from 0.5 mg to 0.5–0.6 mg (P 0.004).

Although a standard unprotected dose of 6-AN (0.4 or 0.5 mg depending on weight) produced signs of distress in mice within 6 to 12 hours (Pinsky and Fraser, 1959), the same dose of 6-AN, followed two hours later by a "single protective dose" of nicotinamide (0.15 mg), did not have any effect on the mother, but was highly teratogenic to developing embryos (Pinsky and Fraser, 1960). When a single protective dose of nicotinamide was given simultaneously with 6-AN there was no increase in malformations or resorptions,
suggesting that the embryos had been protected against the teratogen. However, when the same amount of nicotinamide was given two hours after the teratogen there was a sharp increase in both resorptions and malformations. To determine if this meant that a two hour deficiency of 6-AN was teratogenic, or that a single protective dose of nicotinamide did not completely inhibit the effect of the 6-AN used, a "double protective dose" of nicotinamide (0.3 mg) was given two hours after 6-AN to see if this would result in a lower frequency of malformations than a single protective dose, as would be expected if the latter possibility were the case. In A/Jax x C57BL/6 embryos treated on day 11 1/2 the resorption and cleft palate frequencies were the same after a double dose as after a single dose of nicotinamide given two hours after 6-AN. Thus, for this cross, on this gestational day, a single dose of nicotinamide is as effective as a double dose in counteracting the teratogenic effect of 6-AN. However, after treatment on day 9 1/2 the frequency of cleft lip and cleft palate and of hind limb defects was much lower after
a double dose of nicotinamide than after a single
dose given two hours after the 6-AN. The
frequency of resorption sites is approximately
the same for both doses of nicotinamide. It
would seem that the nicotinamide requirements
of the embryo-mother system studied appear to
vary from day to day.

The high frequency of malformations,
even after a double protective dose of nico-
tinamide, suggested that a two hour exposure
to the inhibiting effect of 6-AN was enough
to cause irreversible damage to developmental
processes. Thus the accuracy with which one
could define the "critical period" during which
6-AN acts to produce malformations in various
developing organ systems was greatly improved.
Using short-term exposures to 6-AN at precisely
known gestational stages, and studying the
resulting teratological effects, Pinsky and
Fraser (1960) observed hind limb defects and
cleft lip with cleft palate after treatment
on day 9 1/2, no malformations, but a high re-
sorption rate, after treatment on day 10 1/2, and
cleft palate after treatment on day 11 1/2.

Goldstein et al. (1963) used the
method just described for timing the exposure of embryos to the effects of 6-AN to demonstrate that the genetic basis for a strain difference in the frequency of induced malformations differs for different organs in animals of the same genotype, and to show that these differences are, in part, determined by cytoplasmic factors. Mice of the A/Jax (A) and C57BL/6J (C) strains were given intramuscular injections of 6-AN (19 mg/kg), followed two hours later by a protective injection of nicotinamide (7.3 mg/kg) on days 8½ to 14½ of gestation. A variety of malformations was produced, depending on the day of treatment, but only vertebral fusions and cleft palate were discussed in detail.

Maternal treatment with 6-AN on day 9½ of gestation resulted in a maximum frequency of vertebral fusions in the thoracic and lumbar regions of both strains; however, the frequency of vertebral fusions was higher in offspring of the A strain (89%) than in those of the C strain (56%), indicating that there are genetically-determined differences in response to the teratogen. The complex nature of these
differences was revealed by the reciprocal cross difference in the F₁ hybrids of the two strains. The frequency of induced vertebral fusions was higher in the C x A (female written first) hybrids (67%) than in the A x C hybrids (45%), a patroclinous reciprocal cross difference.

Results of treatment on day 13½ showed a peak frequency of cleft palate in both strains. However, the A strain embryos had a higher frequency (76%) than those of the C strain (11%). Again, there was a reciprocal cross difference in the F₁, the frequency of induced cleft palate being higher in A x C hybrids (36%) than in C x A hybrids (4%), but this time it was matroclinous.

Since treated embryos from the crosses A x A and A x C have different cleft palate frequencies, but the same type of mother, the embryo's genotype must be important in determining its response to this teratogen. On the other hand, the treated offspring from the cross A x C have a higher frequency of cleft palate than the genetically identical offspring from the C x A cross, so teratogenic susceptibility must also be influenced by the nature of the mother. In
the backcross to the A strain, in which both the maternal and fetal genotypes were identical, the AC x A embryos were more susceptible to cleft palate (23.7%) after 6-AN than the CA x A embryos (5.5%). Since the embryos from these crosses differed in the origin of their maternal cytoplasm, this suggested that there are factors in the A cytoplasm which increase the embryo's susceptibility to the teratogenic activity of 6-AN on day 13 1/2.

Goldstein et al. (1963) feel that embryo genotype and maternal cytoplasmic factors are not the only determinants of susceptibility to cleft palate after treatment with 6-AN on day 13 1/2. If they were, one would expect the cleft palate frequency to be higher in embryos from the cross AC x A than in those from the A x C mating, since the former have more "susceptible" genes than the latter. However, the cleft palate frequency was, if anything, lower in the AC x A cross (24%) than in the A x C cross (36%). It was concluded that the maternal constitution must also play a role in determining susceptibility to treatment, i. e. the embryo appears to be more resistant to 6-AN when growing in an AC uterus
than in an A uterus.

Since the reciprocal cross difference in the frequency of vertebral fusions and cleft palates were in opposite directions, the hereditary factors influencing susceptibility to this teratogen appear to differ for different organ systems in animals of the same genotype. These differences appear to be partly cytoplasmic in nature.

The effects of 6-AN on embryonic development in the Long-Evans rat were extensively studied by Chamberlain and Nelson (1962, 1963a, 1963b). A single intraperitoneal injection (8 mg/kg body weight) any day during the second and third weeks of pregnancy resulted in embryonic death or abnormal development in 65-99% of implantation sites by day 21. Injection on day 9 resulted in 99% fetal death, the incidence of mortality decreasing to 13% following injection on day 15, then increasing to 79% in rats treated on day 20 -- only 24 hours before autopsy. The frequency of abnormal young was high (47-96%) in all groups, but no pattern of abnormalities could be detected, since practically all systems were affected to some extent. Administration
of the antimetabolite once during the second week of gestation — the so-called "critical period" of development in the rat — produced a high incidence of defects previously reported by other investigators using 6-AN on day 10, 11, or 12 of gestation; namely, skeletal defects, cleft palate, cleft lip and exomphalos (Murphy et al., 1957; Murphy, 1960). Additional abnormalities noted included severe defects of the central nervous system and eyes, such as hydrocephalus and posterior lenticular, as well as urogenital and vascular anomalies (Chamberlain and Nelson, 1963, 1963b). The high incidence of embryonic mortality and abnormalities found after 6-AN treatment during the third week of pregnancy were interesting because few metabolic inhibitors have been demonstrated to affect embryonic development when instituted late in gestation.

Cleft palate was observed in the offspring of rats injected once from the eleventh to sixteenth day of pregnancy, the maximum incidence (95%) resulting from treatment on day 13. Both complete and anterior cleft palates were observed in a few embryos (4%).
from mothers injected with 6-AN on day 16 of gestation -- normal palate closure occurs during day 16 and 17 in the Long-Evans rat. The cleft palates observed in this study were associated with "growth retardation of the mandible and failure of descent of the tongue." Retardation of shelf movement was suggested by the small number of anterior clefts observed after day 16 treatment with 6-AN.

Chamberlain and Nelson (1963a) also fed pregnant rats a purified niacin-deficient diet containing 6-AN (100 mg/kg of diet) on days 7-9, 8-10, 9-11, or 10-13 of pregnancy. Ingestion of 1-2 mg of the antimetabolite resulted in embryonic mortality (46%) and a low incidence of malformations. Depending on when the transitory niacin deficiency was administered, 29% to 82% of the implantation sites examined showed either a resorption or an abnormal embryo. Days 8-10 appeared to constitute the period of greatest sensitivity, since only 27% of the rats had living young and 82% of the implantation sites were resorbed or contained an abnormal fetus. Congenital anomalies found included defects of the skeleton, central
nervous system, eye, urinary system, trunk, thyroid, and thymus.

Pregnant rats ate less and lost weight while on the diet containing the antimetabolite. However, fasting control animals during the second week of pregnancy to produce a comparable weight loss did not result in embryonic mortality or malformations.

Administration of 6-AN during the first eight days of gestation also had deleterious effects on developing embryos (Chamberlain, 1963). Regardless of when the antimetabolite was injected during the first week, no implantation sites were observed in 73% of the animals on day 21. The results suggested that 6-AN prevented or delayed implantation. When implantation did occur, subsequent development was greatly retarded -- living young from mothers injected on the first day of pregnancy, though morphologically normal at autopsy, were developmentally equivalent to day 14-15 embryos.

Little is known concerning the precise mechanisms of action of 6-AN in causing abnormalities. Placentas of 14-day embryos following injection of 6-AN on day 13 of pregnancy did not
appear to be different from control placentas. Preliminary assays for inactive pyridine nucleotide coenzymes were carried out on treated fetal tissues. Chromatographic and fluorescent study of tissue homogenates indicated that 18 to 24 hours after injection of 6-AN into the mother, 6-AN analogs of NAD and NADP were present in the embryo (Chamberlain and Nelson, 1963b).

In 1965 Goldstein et al., employing the method described above (Pinsky and Fraser, 1960) for producing a two hour nicotinamide deficiency in the mouse, demonstrated that a single intramuscular dose of 6-AN given to pregnant A/Jax mice on day 10 1/2 of gestation killed a high proportion of the embryos (86.6%). Most of the survivors (81.8%) had a congenital cleft lip, but it was not clear if this defect had been induced by the treatment, or if the embryos destined to have spontaneous cleft lip (about 15% in the A/Jax strain) had been spared. Treatment on day 9 1/2 or 11 1/2 resulted in lower resorption frequencies (about 40%) than on day 10 1/2, and there was no significant indication of a preferential survival of cleft lip embryos. Following a double dose of 6-AN
on day $11^{1/2}$, the resorption frequency was raised (82.8%) but there was no excess of cleft lip embryos at term.

A differential resistance of cleft lip embryos to 6-AN on day $10^{1/2}$ was therefore postulated. Various treatments were given on day $10^{1/2}$, and in each case, whatever the resorption frequency, the number of cleft lip embryos was approximately what would be expected if all the spontaneous cleft lip embryos had survived. Goldstein et al. (1965) concluded that A/Jax embryos destined to have a congenital cleft lip were physiologically different from normal embryos, and that this hypothetical difference made them resistant on day $10^{1/2}$ to a dose of 6-AN that killed their normal litter mates. Since the lip has not begun to form on day $10^{1/2}$, the authors are postulating a physiological difference between normal and cleft lip animals before there is a detectable anatomical difference.

G. Action of 6-AN on the cell:

Kligerman and Shapiro (1957) claimed that combination therapy using 6-AN and irradiation increased tumor regression in mice. With a
possible clinical use in mind, Dewey and Hawes
(1963) tested the effect of 6-AN on the radio-
sensitivity of human fetal liver cells in culture
using colony-forming ability as a measure of
survival. In the absence of 6-AN the dose of
radiation giving 37% survival of colonies was
140 rads. Adding 6-AN (2mM) to the cultures
shortly before irradiation seemed to sensitize
cells to radiation damage, in that there was a
decreased number of surviving colonies after
the same dose of X-rays.

However, in vitro experiments on
Ehrlich ascites tumor cells of the mouse, using
abnormal telophase figures as a criterion of
damage, produced no evidence of sensitization,
suggesting that the results might not be due
to radiosensitization. These results indicated
a difference in the effect of 6-AN on radio-
sensitivity as measured by chromosome damage
on the one hand, and reproductive death on the
other.

The essential difference between the
two types of experiments done was that in the
former experiment 6-AN was added to cells still
in suspension, and in the latter experiment it
was added to cells that were attached to a surface. It was concluded that 6-AN reduced the plating efficiency of suspended cells, but did not sensitize them to irradiation damage.

Ingalls et al. (1963) reported that 6-AN treatment on day 13 could cause chromosomal anomalies such as polyploidy and fragmentation as accompaniments of cleft palate formation in mice. Microscopic examination of cell cultures of palatine tissue, muscle, skin, and limb fragments from cleft palate animals revealed chromosomal aberrations in 38.7% of the cells observed, as compared with 5.2% in cells from untreated controls. In a later study (1964) the same group used 6-AN on day 11 1/2 to produce 68% cleft palate in mice. However, after this treatment more chromosomal aberrations were found in cells cultured from treated embryos with normal palates (37.1%) than in cells from treated embryos with cleft palates (31.6%). Only 5.1% of cell complements from untreated control embryos showed any aberrations. Therefore, it appeared that 6-AN could induce chromosomal aberrations in treated embryos, but there was no causal relationship between these abnormal
chromosome complements and cleft palate production with 6-AN.

H. Biological effects of 6-AN:

The toxicity of 6-AN has been extensively studied in laboratory animals (rats, rabbits, cats, dogs, mice, etc.). In all species investigated, the most prominent feature of intoxication was that of central nervous system damage, manifested by motor weakness and hind-limb paralysis progressing to quadriplegia, coma, and death (Johnson and McColl, 1955; Baserga et al., 1956; Halliday, 1957; McColl et al., 1957; Sullivan and Strong, 1958). Pathologically, specific degenerative changes in the grey matter of the anterior horns of the spinal cord and the nuclei of the brain stem have been demonstrated (Sternberg and Philips, 1955, 1959; Wolf and Cowen, 1959; Schotland et al., 1965). In addition, splenic involution (atrophy of lymph follicles), adrenal, hepatic, and testicular focal degenerative changes, and atrophic lesions of the buccal and gastrointestinal mucosa have been observed (Baserga et al., 1956; Morsiani and Soffritti, 1956; Morsiani and Califano, 1958; Esposito and Patronio, 1958; Sternberg and Philips, 1958). Hematologic depression in the form of rapid disappearance of reticulocytes and reduction in the
number of circulating leukocytes -- particularly lymphocytes -- has been described in mice (Baserga et al., 1956; Morsiani and Soffritti, 1956; Morsiani and Califano, 1958). Modifications of the blood level of some enzymes have been observed in rabbits intoxicated with 6-AN (Petronio et al., 1958), and an inotropic and negative chronotropic action has been observed on isolated rat and rabbit hearts perfused with 6-AN (Inesi and Garagnani, 1958).

I. Clinical Experience with 6-AN:

Patients showed a variable response to treatment with 6-AN, but the more depleted the patient nutritionally, the more severe and rapid the toxic response. This was due either to lower initial levels of nicotinamide and tryptophan, or to impaired detoxification of drugs in the liver (Herter et al., 1961).

The toxic response to 6-AN has two clinical forms: prolonged administration in low doses leads to mixed B-complex vitamin deficiency and higher doses have a direct action on the central nervous system, causing lethargy and disorientation. There is no motor disability, as in all acute toxicity experiments with laboratory animals, but this is probably due to lower doses. It appears unlikely that 6-AN will have
clinical value because in safe doses it has no effect on tumors and in higher doses it causes neurological disorders (Sternberg and Philips, 1959).

Kligerman and Shapiro (1957) and Martin et al. (1958) claimed that combination radiotherapy and chemotherapy (6-AN and 6-mercaptopurine) caused tumor regression in mice, but this treatment has not proved to be clinically practical.
III. MATERIALS AND METHODS

A. Mice and Maintenance:

The mice used in the experiments to be described below were of the A/Jax and C57BL/6J strains. Both strains were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine, at 6-8 weeks of age. A/Jax mice, which have the genetic constitution \texttt{aa bb cc}, have been maintained for more than 133 generations by brother-sister matings (Mouse News Letter, 1965a). The C57BL/6J animals, which are genotypically \texttt{aa}, have been inbred by brother-sister mating for over 87 generations (Mouse News Letter, 1965b).

The animals were housed in plastic cages in an air-conditioned, constant-temperature room at 70°Farenheit. A cycle of sixteen hours light and eight hours darkness was in effect throughout the year.

All the mice used in the kinetics experiments received, \textit{ad libitum}, a diet of Purina Mouse Breeder Chow and water. The diet of the mice used in the cytoplasmic factor experiment consisted of Purina Lab Chow and water, supplemented once a week with whole wheat bread, milk, and lettuce.
B. Timing of embryos:

The males and females were kept in separate cages. Twice a week in the early evening a male was placed in a breeding cage with five mature females (3 to 4 months and 20-26 gms). The male was removed the following morning and the females were examined for vaginal plugs. Those showing signs of insemination were weighed and removed to other cages -- three females per cage. For the purpose of estimating embryonic age, fertilization was assumed to occur at 2 am (Runner and Ladman, 1950; Snell et al., 1940). The day the vaginal plug was observed was called day 0 of gestation (e.g. 2 pm of that day the embryos were assumed to have an age of 0 days, 8 hours).

C. Treatment of pregnant females:

Most of the animals used in these experiments were treated on day 13½ unless otherwise stated. At this time pregnant females were weighed, treated and isolated until day 18½. The specific treatment varied from experiment to experiment but the standard treatment consisted of an initial injection of 6-aminonicotinamide (Horner Laboratories) at 2 pm E.S.T., followed by an injection of nicotinamide (Horner Laboratories) two hours later. Both compounds were dissolved in sterile distilled water. They were
administered intraperitoneally using a Hamilton 0.5 cc Microlite Syringe.

On day 18½ of gestation the animals were killed and the offspring examined. The positions of the embryos and resorption sites in the uterus were recorded and the embryos examined for cleft palate and other aberrations. Only litters with four or more viable embryos were included in the results.

D. Treatment of data:

To circumvent the difficulties inherent in binomial data, lack of independence of the mean and variance -- particularly important where percentages cover a wide range -- angular transformation (arc sine) was used. This method was selected empirically by Schlager and Dagg (1955) after testing several transformations because it distorted the data least when the results were compared to the straight percentages.

The data was weighted by giving the arc sine calculated for each litter a weight dependent on the size of the litter, (the larger the litter, the more valid the data, and consequently, the more weight it should carry). This also equalizes the contribution of each embryo to the total. The data were corrected by substituting 1/4N for zero incidence.
of cleft palate and $\frac{4N-1}{4N}$ for 100% cleft palate, where $N$ is the litter size.

To test the sigmoidicity of the dose-response curves the probit transformation was used. This transformation is widely employed for this purpose in biological assays where response is all or none. The weighted arc sine proportions of cleft palate among the experimental animals at various doses of 6-AN were converted into probits, and these probits were plotted against the dose of 6-AN. The points are expected to fall on a straight line, within the limits of sampling error, if the curve is sigmoid (Mather, 1951). A simple regression analysis was used to elucidate this relationship statistically.

E. Amniotic fluid measurement:

Pregnant A/Jax females were injected, intraperitoneally, with 9.5 mg/kg of 6-AN dissolved in water in a concentration of 2.25 mg/cc on day 13 1/2 of gestation. The females were killed on day 16/0 and 16/2.

The intact uteri were quickly removed, wrapped in cellophane, pinned to a flat surface and frozen at -30 C. When measurements were to be made the uterus was removed from the freezer and gently thawed by a stream of warm air before the uterine wall
was peeled away. The embryos, in their sacs, were then returned to the freezer to await weighing. After weighing, each embryo was placed in a moist chamber to thaw. When the amniotic fluid had thawed the sac was punctured, the fluid was blotted with filter paper and the embryo and membranes were re-weighed. The difference in the two weighings was taken to be the weight of the amniotic fluid. After weighing, the embryo was fixed in Bouin's fluid for 24 hours and then the palate was examined.
IV. RESULTS AND DISCUSSION

It was stated in Materials and Methods that pregnant females of the C57BL/6J and A/Jax strains were treated with 6-AN on day 13\(\frac{1}{2}\) of gestation. It was necessary before starting the series of experiments designed to elucidate the kinetics of 6-AN teratogenesis with respect to cleft palate, and the relation of strain differences in response thereto, to define as precisely as possible the "critical period" for cleft palate production with this teratogen in both strains.

Goldstein et al. (1963) and Goldstein (1964) had reported that a two hour maternal inactivation of nicotinamide on day 12\(\frac{1}{2}\), 13\(\frac{1}{2}\), or 14\(\frac{1}{2}\) produces a peak frequency of cleft palate on day 13\(\frac{1}{2}\) of gestation in both A/Jax and C57BL mice. However, the palates of A/Jax mice, which have a high frequency of cortisone- and 6-AN-induced cleft palate, close later than those of C57BL mice, which have a low frequency of cortisone- and 6-AN-induced cleft palate (Walker and Fraser, 1956; Goldstein et al., 1963; Goldstein, 1964). The earlier cortisone work
had suggested a relationship between time of normal palate closure and susceptibility to cortisone treatment. This was investigated by studying palate closure patterns in embryos of the A/Jax and C57BL strains. Trasler (1958) found that palate closure for the A/Jax strain is from day 14/18 (14 days 18 hours) to 15/8 and occurs later and more quickly than in the C57BL strain where it is from day 14/8 to 15/10. These results agreed with those of Walker and Fraser (1956), who found that palate closure began as much as 10 to 12 hours earlier in the C57BL strain than in the A/Jax strain. However, since there is a great variability in the development of embryos from different litters of the same chronological age, and even of embryos within the same litter, Walker felt that morphological stage of development was a better criterion of age of the embryo than chronological time. The developmental age at which palate closure occurred also differed in the A/Jax and C57BL strains -- palate closure began later and proceeded more quickly in A/Jax than C57BL mice (Walker and Fraser, 1956; Trasler, 1958).

These studies led to the argument that
the difference in cleft palate frequency between the two strains after 6-AN treatment on day $13^{1/2}$ did not represent a valid comparison, since the critical period for palate closure in the C57BL strain might have occurred half a day earlier. This was a valid criticism which had to be answered before the A/Jax strain could be considered "susceptible" and the C57BL strain "resistant" to 6-AN treatment.

A. Critical Period Experiment:

A two hour exposure of pregnant A/Jax mice to the action of a $3/4$ dose of 6-AN on day $12^{1/2}$, $13$, $13^{1/2}$, or $14$ of gestation resulted in the production of cleft palate. The frequency was highest following treatment on day $13^{1/2}$ (67%) (see figure 2) and was significantly larger than that observed following exposure to the teratogen on day $12^{1/2}$ (44%) ($X^2=7.67$, d.f. = 1, $P<0.01$), day $13$ (30%) ($X^2=21.80$, d.f. = 1, $P<0.005$), and day $14$ (32%) ($X^2=16.99$, d.f. = 1, $P<0.005$) (table 1).

These data confirm the reports of Goldstein et al. (1963) and Goldstein (1964) that the critical period for the production of cleft palate with 6-AN in the A/Jax strain is day
<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Dose</th>
<th>Strain</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D 12 1/2</td>
<td>3/4</td>
<td>A/Jax</td>
<td>11</td>
<td>38</td>
<td>85</td>
<td>43.7%</td>
</tr>
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<td></td>
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<td>12</td>
<td>80</td>
<td>13.1%</td>
</tr>
<tr>
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<td>3/4</td>
<td>A/Jax</td>
<td>13</td>
<td>32</td>
<td>100</td>
<td>29.9%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C57BL</td>
<td>11</td>
<td>6</td>
<td>72</td>
<td>8.9%</td>
</tr>
<tr>
<td>D 13 1/2</td>
<td>3/4</td>
<td>A/Jax</td>
<td>11</td>
<td>65</td>
<td>100</td>
<td>67.2%</td>
</tr>
<tr>
<td></td>
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<td>C57BL</td>
<td>11</td>
<td>50</td>
<td>86</td>
<td>59.7%</td>
</tr>
<tr>
<td>D 14</td>
<td>3/4</td>
<td>A/Jax</td>
<td>10</td>
<td>26</td>
<td>77</td>
<td>31.8%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C57BL</td>
<td>12</td>
<td>13</td>
<td>88</td>
<td>14.2%</td>
</tr>
</tbody>
</table>

Table 1
Number and weighted arc sine percentages of 6-AN-induced cleft palates in the A/Jax (3/4 dose) and C57BL (1 dose) strains tabulated by half day intervals.
Figure 2

Critical period in the A/Jax and C57BL strains by half day intervals.
The dose of 6-AN had to be changed before the same procedure could be carried out on the C57BL strain. The dose of 6-AN used in the A/Jax strain (3/4 dose) did not produce high enough cleft palate frequencies for meaningful analysis, while twice the dose used in the A/Jax strain (1 1/2 dose) produced 100% cleft palate on day 13 1/2, which was also undesirable (table 2). Finally, a single standard dose of 6-AN was found to produce cleft palate frequencies in the C57BL strain suitable for statistical analysis. It might be noted, however, that all three doses of 6-AN showed peak frequencies of cleft palate on day 13 1/2 in the C57BL strain (see figure 3).

When the experiment was performed with the C57BL strain using one dose of 6-AN on day 12 1/2, 13, 13 1/2, or 14 of gestation, the frequency of cleft palate was again highest following treatment on day 13 1/2 (60%) (see figure 2) and was significantly larger than that observed after treatment on day 12 1/2 (13%) ($X^2 = 32.96$, d.f. = 1, $P < 0.005$), day 13 (9%) ($X^2 = 44.64$, d.f. = 1, $P < 0.005$), and day 14 (14%) ($X^2 = 35.41$, d.f. = 1, $P < 0.005$) (table 1).
<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Dose</th>
<th>Strain</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D 12½</td>
<td>3/4</td>
<td>C57BL</td>
<td>12</td>
<td>1</td>
<td>93</td>
<td>3.7%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C57BL</td>
<td>11</td>
<td>12</td>
<td>80</td>
<td>13.1%</td>
</tr>
<tr>
<td>D 13</td>
<td>3/4</td>
<td>C57BL</td>
<td>11</td>
<td>2</td>
<td>76</td>
<td>5.0%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C57BL</td>
<td>11</td>
<td>6</td>
<td>72</td>
<td>8.9%</td>
</tr>
<tr>
<td></td>
<td>1½</td>
<td>C57BL</td>
<td>10</td>
<td>68</td>
<td>78</td>
<td>88.3%</td>
</tr>
<tr>
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<td>C57BL</td>
<td>10</td>
<td>6</td>
<td>69</td>
<td>9.0%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C57BL</td>
<td>11</td>
<td>50</td>
<td>86</td>
<td>59.7%</td>
</tr>
<tr>
<td></td>
<td>1½</td>
<td>C57BL</td>
<td>11</td>
<td>82</td>
<td>84</td>
<td>95.5%</td>
</tr>
<tr>
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<td>C57BL</td>
<td>13</td>
<td>4</td>
<td>105</td>
<td>5.1%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C57BL</td>
<td>12</td>
<td>13</td>
<td>88</td>
<td>14.2%</td>
</tr>
<tr>
<td></td>
<td>1½</td>
<td>C57BL</td>
<td>8</td>
<td>18</td>
<td>57</td>
<td>29.7%</td>
</tr>
</tbody>
</table>

Table 2
Number and weighted arc sine percentages of 6-AN-induced cleft palates in the C57BL strain tabulated by half day intervals and by dose.
Figure 3

Critical period in the C57BL strain at different dose levels.
Day of Treatment with 6-AN

- % CP (weighted arcsine)

C57BL (1½ dose)
C57BL (1 dose)
C57BL (¾ dose)
The results of this preliminary study indicated that day $13^{1/2}$ was the critical period for cleft palate production in both the C57BL and A/Jax strains following a two hour inactivation of nicotinamide by 6-AN on day 12$1/2$, 13, 13$1/2$, or 14 (table 1). Thus, although palate closure commences half a day earlier in C57BL mice than in A/Jax mice, the period of maximum sensitivity of the palate to this teratogen was shown to be the same in both strains. In addition, the C57BL strain required more 6-AN than the A/Jax strain to produce a comparable cleft palate frequency, so it may correctly be termed a "resistant" strain.

Having clarified this point, it was now possible to proceed with the kinetic study of 6-AN, which we hoped would reveal why the palate closure mechanism of the C57BL strain is resistant to the action of 6-AN, while that of the A/Jax strain is susceptible.

Goldstein (1964) investigated this problem and suggested that "the strain difference in the frequency of cleft palate production on day $13^{1/2}$ due to a 2 hour exposure to 6-AN is due to a higher level of nicotinamide in the C57BL than in the
A/Jax strain." This hypothesis was based on a series of experiments in which pregnant C57BL mice were given a single intramuscular dose of 6-AN on day $13^{1/2}$ of gestation, followed two hours later by an injection of distilled water or a single dose of nicotinamide. The results of treating C57BL mice with a single dose of 6-AN followed by a single protective dose of nicotinamide (10.8% cleft palate), when compared with those obtained after similar treatment of A/Jax mice (76% cleft palate), led to the conclusion that the rate of incorporation of 6-AN was lower in the C57BL strain. Longer exposure of C57BL mice to the teratogenic action of 6-AN supported this observation, since the cleft palate frequency in unprotected mice rose to 46.6% after a single dose of 6-AN.

Goldstein postulated that this decreased rate of incorporation of 6-AN might be due to either a decreased rate of synthesis of NAD, or a larger nicotinamide pool in the C57BL strain as compared to the A/Jax strain. The first alternative is not supported by evidence that indicates that 6-ANAD analogs are formed by exchange reactions between the nicotinamide moiety of NAD and 6-AN, rather
than by synthetic reactions (Johnson and McColl, 1956; Dietrich et al., 1958). This gives the second postulated explanation added weight, since the rate of incorporation of an antimetabolite in an exchange reaction is proportional to the relative concentration of the metabolite and the antimetabolite (Woolley, 1952).

When a double dose of 6-AN was administered to a second group of pregnant C57BL mice and protected two hours later by a single dose of nicotinamide, the cleft palate frequency rose considerably to 73.1%. A single dose of nicotinamide was sufficient, however, to counteract the action of a double dose of 6-AN in C57BL mice, since there was no reduction in cleft palate frequency (72.2%) when a double dose of 6-AN was followed by a double dose of nicotinamide. These observations were cited by Goldstein (1964) as additional support for his hypothesis of a larger nicotinamide pool in the C57BL strain. He felt that the relative concentrations of nicotinamide and 6-AN in C57BL mice, following a single dose of 6-AN, were such that nicotinamide could compete successfully with 6-AN for incorporation into NAD. However, a double dose of 6-AN was able to overcome the high concentration of nicotinamide present in the tissues of C57BL mice and compete in the reaction which forms
non-physiological NAD analogs. Goldstein concluded his discussion of this hypothesis by stating that another possibility is a genetic difference in the affinity of 6-AN for the NAD and NADP molecules in these two strains of mice, which results from minor interstrain differences in the coenzymes.

B. Kinetic study of 6-AN:

This kinetic study of 6-AN teratogenesis with respect to cleft palate formation was undertaken in a biochemically naive attempt to determine if the strain differences in response to 6-AN reported above reflected differences in (1) nicotinamide pools, (2) affinities for enzyme(s) involved in the formation of NAD analogs, or (3) coenzyme turnover rates.

1. Dose-response curves

Palate closure may be viewed essentially as a matter of the "intrinsic shelf force" overcoming the resistance of the tongue (Walker and Fraser, 1956). If this hypothesis is correct, the shelf force is influenced by both maternal and fetal genes, and by 6-AN and other environmental factors. The resistance of the tongue is probably also influenced by many factors, but they remain unknown for lack of investigation. The interaction of these two forces determines when the shelves
assume a horizontal position. Embryos differ in the
time at which the shelves become horizontal (random
biological variation), so that a frequency distribution
of the times when the shelves move from their vertical
position on either side of the tongue and reach their
horizontal position above the tongue will be a normal,
continuous frequency distribution.

Cleft palate occurs if movement of the
shelves has been delayed so that after they come up
they are not able to touch and fuse. The delay in
the movement that causes the shelves to come up may
be defined as the threshold which divides the con-
tinuous distribution of times of shelf movement into
two discontinuous parts -- on one side of the threshold
the palate is closed, on the other side it is cleft.
This phenomenon, where a threshold breaks a continuous
distribution into a discontinuous distribution which
behaves genetically as if it reflected an underlying
continuous distribution of some variable, is what
Grunenberg (1952) has called "quasi-continuous
variation" (Fraser et al., 1957).

A characteristic of quasi-continuous
variations which is relevant to a discussion of the
dose-response curve of a teratogen was pointed out
by Fraer (1965). Often teratogens act by shifting the distribution of a developmental process so that a number of individuals are pushed over a threshold and are abnormal. If the effect of a teratogen on a distribution increases linearly with increasing dose, the increase in the proportion of abnormal individuals with increasing dose will be sigmoid. Thus, we would expect a sigmoid dose-response curve for cleft palate frequency following administration of increasing amounts of 6-AN.

Pregnant female mice of the A/Jax and C57BL strains were exposed to various unprotected doses of 6-AN on day 13½ of gestation and their litters examined for cleft palate just before birth (tables 3 and 4). When the proportion of embryos with cleft palate was plotted against the dose of 6-AN, the dose-response patterns for both strains appeared to be sigmoid (see figure 4). To test the sigmoidicity of these curves the probit transformation was used. This transformation is widely employed for this purpose in biological assays where response is all or none. When the probit transformations were plotted against dose, a simple regression analysis showed
<table>
<thead>
<tr>
<th>Dose of 6 AN</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/8</td>
<td>10</td>
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<td>76</td>
<td>4.9%</td>
</tr>
<tr>
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<td>11</td>
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<td>98</td>
<td>10.8%</td>
</tr>
<tr>
<td>3/8</td>
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<tr>
<td>5/8</td>
<td>12</td>
<td>86</td>
<td>90</td>
<td>94.1%</td>
</tr>
<tr>
<td>3/4</td>
<td>13</td>
<td>93</td>
<td>93</td>
<td>96.6%</td>
</tr>
</tbody>
</table>

Table 3
Number and weighted arc sine percentages of cleft palate A/Jax embryos treated with various unprotected doses of 6-AN on day 13 1/2 of gestation.
## Table 4

Number and weighted arc sine percentages of cleft palate C57BL embryos treated with various unprotected doses of 6-AN on day 13 1/2 of gestation.

<table>
<thead>
<tr>
<th>Dose of 6 An</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
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<tbody>
<tr>
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<td>12</td>
<td>2</td>
<td>103</td>
<td>3.9%</td>
</tr>
<tr>
<td>1/2</td>
<td>12</td>
<td>6</td>
<td>92</td>
<td>6.4%</td>
</tr>
<tr>
<td>5/8</td>
<td>12</td>
<td>10</td>
<td>91</td>
<td>10.0%</td>
</tr>
<tr>
<td>3/4</td>
<td>12</td>
<td>48</td>
<td>88</td>
<td>48.8%</td>
</tr>
<tr>
<td>7/8</td>
<td>12</td>
<td>66</td>
<td>83</td>
<td>82.8%</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>76</td>
<td>76</td>
<td>96.7%</td>
</tr>
</tbody>
</table>
Figure 4
Dose-response curves for the A/Jax and C57BL strains.
Figure 5

Dose-response curves for the A/Jax and C57BL strains using the probit transformation (straight percentages and weighted arc sine percentages).
a good linear correlation for both the C57BL (t 8.01, P .01) and A/Jax (t 9.06, P .01) strains suggesting that the dose-response curves of the original data were sigmoid.

It was hoped that these dose-response curves would give us some information about the relative size of the nicotinamide pools in the C57BL and A/Jax strains, and/or the relative affinities of exchange reaction enzymes (NADases) for 6-AN in these strains. The similar appearance of the curves was taken as an indication that the NADase affinities for 6-AN in both strains were approximately the same, and that a larger nicotinamide pool in the C57BL mice shifted that curve to the right until enough 6-AN had been added to the system to "overcome" the extra nicotinamide. However, an expert on enzyme kinetics (Scholefield, personal communication) advised that the only conclusion one should draw from a biological assay of this sort is that it takes more 6-AN to cause cleft palate in the C57BL strain than in the A/Jax strain, as indicated by the fact that the curve for the C57BL strain is shifted to the right in figure 4.
This is not to imply that the original interpretation of the results might not be correct; it merely means that an assay as "crude" as this one cannot distinguish between the alternative explanations proposed, or detect if an interaction between them is responsible for the result.

A second biochemist (Scriver, personal communication) expressed the opinion that the sigmoidicity of the curves need not necessarily be an indication that a quasi-continuous variate was being measured. The sigmoid curves obtained in this experiment could result from the different affinities of nicotinamide and 6-AN for enzymes in the developing palate, and reflect the kinetics of this competitive reaction rather than an underlying continuous distribution interrupted by a threshold. They might also be the result of a combination of both these elements.

The dose-response curves presented here may seem rather steep to those who regularly deal with dose-response relationships of a non-teratological nature. However, a sharp rise in the dose-response curves of chemical teratogens is not uncommon and, in fact, appears to be the rule (Wilson, 1964). It has almost become
axiomatic that most teratogenic agents can be tolerated in low dosage without any recognizable effect on the developing embryo, but rather quickly become teratogenic, and lethal, to all embryos as higher dosages are reached (Wilson, 1964). Between these ranges of normality and lethality there exists a narrow teratogenic zone of dosages in which embryos survive with varying degrees of teratogenic involvement. The C57BL dose-response curve indicates just how narrow this zone is, since it rises from 6.4% cleft palate at a half dose of 6-AN to 100% cleft palate at a full standard dose (table 4). The phenomenon of a precipitous dose-response curve is not unique to chemical teratogens. Wilson (1954) demonstrated that doubling the dose of X-rays given to an embryo could increase the incidence of malformations from none to 100%.

2. How much nicotinamide is equivalent to a given amount of 6-AN?

The role of exogenous nicotinamide in neutralizing the effect of 6-AN and a variety of other teratogens is well established in several systems (Landauer, 1948; Landauer, 1949; Zwilling and DeBell, 1950; Landauer, 1953; Landauer, 1957; Murphy et al., 1957; Pinsky and Fraser, 1959, 1960).
To find out whether the amount of nicotinamide needed to forestall the teratogenic action of 6-AN, using cleft palate as an indicator, was the same in the C57BL and A/Jax strains the following experiment was performed. A "standard unprotected dose" of 6-AN -- one which will give close to 100% cleft palate (unprotected) -- was injected simultaneously with varying amounts of nicotinamide into pregnant mice of both strains on day 131/2 of gestation. The dose-response curves (tables 3 and 4) revealed that there was no one dose of 6-AN that could be used in both the A/Jax and C57BL strains to produce just under 100% cleft palate -- or any other frequencies which would permit meaningful analysis of the results. The standard unprotected dose of 6-AN selected for use in the A/Jax strain was 5/8 of a standard (protected) dose, which produces 94% cleft palate; in C57BL mice 7/8 of a standard dose of 6-AN, which produces 82.8% cleft palate, was chosen as the standard unprotected dose. Various fractions of the corresponding standard dose of nicotinamide were injected simultaneously with the 6-AN. The smallest dose of nicotinamide to provide complete protection against the teratogenic action of 6-AN, when
they were injected simultaneously, was called the "equivalent" dose of nicotinamide.

A preliminary experiment was run in both strains to locate roughly the dose range of nicotinamide which provided full protection against the standard unprotected dose of 6-AN. Tables 5 and 6 present the results obtained from this study. In the A/Jax strain about 3/4 of the standard dose of nicotinamide usually used to protect against standard doses of 6-AN was needed to provide complete protection, although lower doses provided partial protection (see table 5 and figure 6). The amount of nicotinamide necessary to forestall completely the teratogenic action of 6-AN in the C57BL strain was approximately 3/8 to 1/2 the standard dose (see table 6 and figure 6).

Having established in an approximate way the doses at which cleft palate appeared in both strains, a second experiment was performed, using larger numbers of mice around these points to determine the critical dose of nicotinamide in the A/Jax and C57BL strains. From the data in tables 7 and 8 it is evident that 3/4 of a 5/8 standard dose of nicotinamide (15/32), or
<table>
<thead>
<tr>
<th>Dose of nicotinamide</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>27</td>
<td>30</td>
<td>81.9%</td>
</tr>
<tr>
<td>1/8</td>
<td>5</td>
<td>26</td>
<td>37</td>
<td>71.0%</td>
</tr>
<tr>
<td>1/4</td>
<td>5</td>
<td>25</td>
<td>40</td>
<td>63.6%</td>
</tr>
<tr>
<td>3/8</td>
<td>5</td>
<td>16</td>
<td>42</td>
<td>37.8%</td>
</tr>
<tr>
<td>1/2</td>
<td>5</td>
<td>12</td>
<td>36</td>
<td>31.9%</td>
</tr>
<tr>
<td>5/8</td>
<td>5</td>
<td>8</td>
<td>35</td>
<td>22.3%</td>
</tr>
<tr>
<td>3/4</td>
<td>5</td>
<td>1</td>
<td>34</td>
<td>5.1%</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>39</td>
<td>3.2%</td>
</tr>
</tbody>
</table>

Table 5
Preliminary experiment to determine how much nicotinamide is equivalent to a simultaneously administered 5/8 dose of 6-AN on day 13 1/2 of gestation in the A/Jax strain.
<table>
<thead>
<tr>
<th>Dose of nicotinamide</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2</td>
<td>10</td>
<td>20</td>
<td>76</td>
<td>25.1%</td>
</tr>
<tr>
<td>5/8</td>
<td>10</td>
<td>13</td>
<td>69</td>
<td>17.6%</td>
</tr>
<tr>
<td>3/4</td>
<td>10</td>
<td>1</td>
<td>73</td>
<td>3.9%</td>
</tr>
</tbody>
</table>

Table 6
Second experiment to determine the critical dose of nicotinamide in the A/Jax strain.
<table>
<thead>
<tr>
<th>Dose of nicotinamide</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>29</td>
<td>39</td>
<td>75.3%</td>
</tr>
<tr>
<td>1/8</td>
<td>5</td>
<td>20</td>
<td>38</td>
<td>52.5%</td>
</tr>
<tr>
<td>1/4</td>
<td>5</td>
<td>8</td>
<td>35</td>
<td>21.5%</td>
</tr>
<tr>
<td>3/8</td>
<td>5</td>
<td>3</td>
<td>43</td>
<td>7.7%</td>
</tr>
<tr>
<td>1/2</td>
<td>5</td>
<td>1</td>
<td>33</td>
<td>5.3%</td>
</tr>
<tr>
<td>5/8</td>
<td>5</td>
<td>0</td>
<td>38</td>
<td>3.3%</td>
</tr>
<tr>
<td>3/4</td>
<td>5</td>
<td>0</td>
<td>29</td>
<td>4.3%</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>37</td>
<td>3.4%</td>
</tr>
</tbody>
</table>

Table 7
Preliminary experiment to determine how much nicotinamide is equivalent to a simultaneously administered 7/8 dose of 6-AN on day 13 1/2 of gestation in the C57BL strain.
<table>
<thead>
<tr>
<th>Dose of nicotinamide</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4</td>
<td>10</td>
<td>18</td>
<td>76</td>
<td>21.7%</td>
</tr>
<tr>
<td>3/8</td>
<td>11</td>
<td>7</td>
<td>80</td>
<td>8.7%</td>
</tr>
<tr>
<td>1/2</td>
<td>11</td>
<td>1</td>
<td>78</td>
<td>4.0%</td>
</tr>
</tbody>
</table>

Table 8
Second experiment to determine the critical dose of nicotinamide in the C57BL strain.
Figure 6

Preliminary experiment to determine how much nicotinamide is equivalent to a simultaneously administered dose of 6-AN on day $13\frac{1}{2}$ of gestation in the A/Jax and C57BL strains, and repeat experiments to establish equivalent dose of nicotinamide.
Preliminary experiment
- A Jax (5/8 dose) 6'AN
- C57Bl (7/8 dose) 6'AN

Repeat experiment
- A Jax (5/8 dose) 6'AN
- C57Bl (7/8 dose) 6'AN

CP (weighted arcsine)

Dose of Nicotinamide
3.42 mg/kg body weight of nicotinamide is needed by the A/Jax strain to overcome the effects of a 5/8 dose of 6-AN. However, the C57BL strain needs only 1/2 of a 7/8 standard dose of nicotinamide (7/16), or 3.19 mg/kg body weight of nicotinamide, to forestall the teratogenic activity of a 7/8 dose of 6-AN.

From these data it is evident that the C57BL strain needs less nicotinamide than the A/Jax strain (3.19 mg/kg vs 3.42 mg/kg) to counteract a larger dose of 6-AN (16.63 mg/kg vs 11.88 mg/kg). If the ratio of 6-AN to N is compared for the two strains of mice, an interesting comparison emerges. The ratio is about 1 to 3.5 in the A/Jax strain (3.42 to 11.8), but only 1 to 5.2 in the C57BL strain (3.19 to 16.63). In other words, the A/Jax strain requires one and a half times as much nicotinamide as the C57BL strain.

Once again, it is not possible to draw any conclusions from these results about whether the C57BL strain needs less nicotinamide because it has a larger nicotinamide pool, or lower enzyme affinities for 6-AN than the A/Jax strain.

3. Kinetic study of 6-AN teratogenesis

Pinsky and Fraser (1960) investigated
the protective action of nicotinamide following administration of 6-AN. When a "single protective dose" (0.15 mg) of nicotinamide was given simultaneously with 6-AN on day 9½, 10½, or 11½, there was no appreciable increase in the number of resorptions and malformations, suggesting that a "single protective dose" of nicotinamide completely protects the embryo when given simultaneously with the teratogen. However, the effectiveness of nicotinamide in protecting the embryo against 6-AN declines gradually with increasing time intervals between administration of the two compounds. Thus, when the same dose was given two hours after the teratogen, there was a significant rise in the frequency of both resorptions and malformations.

Goldstein (1964) reported that the frequency of cleft palate in the A/Jax strain on day 13½ was 76.1% following administration of a standard dose of 6-AN and, two hours later, a protective dose of nicotinamide. To find out if the protective dose of nicotinamide used was sufficient to arrest the action of 6-AN after two hours, a number of mice were given a double dose of nicotinamide two hours after injection of 6-AN. There was no significant difference between the
rate of resorption and cleft palate frequency found after a single dose of nicotinamide. When no protective dose of nicotinamide was given the frequency of cleft palate rose to 100%. This established that a standard dose of 6-AN acts for more than two hours if no protective dose of nicotinamide is administered. It also determined that in the A/Jax strain about 80% of the effect of 6-AN as measured by cleft palate frequency has been completed by the end of two hours.

The final experiment performed in this study of the kinetics of 6-AN teratogenesis was designed to investigate the timed interaction of nicotinamide and 6-AN in the A/Jax and C57BL strains and answer the following questions: how long after its injection is 6-AN still effective in causing irreversible developmental damage to the embryo; at what point following administration of 6-AN does nicotinamide lose its protective ability; does prior administration of nicotinamide afford protection against an injection of 6-AN, and if so, for how long; and lastly, are the answers to these questions different in the two strains?

An amount of nicotinamide equivalent
in each strain to a \( \frac{3}{4} \) dose of 6-AN (assuming that the ratio of the requirement is the same at all doses) was calculated from the results of the previous experiment and injected into pregnant females at times varying from 0 to 8 hours before or after administration of a \( \frac{3}{4} \) dose of 6-AN on day 13\( \frac{1}{2} \) of gestation. The results of this experiment are represented graphically in figure 7.

An injection of nicotinamide, given two hours prior to administration of 6-AN, provided almost complete protection against the effect of the teratogen in the C57BL strain, but only partial protection (70\% at two hours) in the A/Jax strain. In fact, a dose of nicotinamide given 8 hours before injection of 6-AN still affords about 50\% protection in the C57BL strain, while in A/Jax mice the ability of a prior injection of nicotinamide to forestall cleft palate production by 6-AN is almost completely lost if it is given four hours before the teratogen.

Simultaneous injection of nicotinamide and 6-AN provided almost complete protection in both strains, as has been previously reported (Pinsky and Fraser, 1960; Goldstein, 1964).

The teratogenic effect of short periods of exposure to 6-AN is quite striking in the A/Jax
Figure 7

Study of the kinetics of 6-AN teratogenesis in the A/Jax and C57BL strains.
Nicotinamide before 6·AN

Nicotinamide after 6·AN

% CP (weighted arcsine)

A/Jax Control

C57BL Control

ACV/66
strain. Over 50% of A/Jax embryos have cleft palates after exposure to 6-AN for only one hour. By the end of a two hour interval between 6-AN and nicotinamide administration, the cleft palate frequency is 100%. It is unfortunate that no dose of 6-AN could be found which would produce less than 100% cleft palate in the unprotected A/Jax controls and high enough cleft palate frequencies for meaningful analysis in the unprotected C57BL controls. Because the dose of 6-AN used in this experiment produces 100% cleft palate in A/Jax mice after two hours, it is not possible to know from this experiment if the teratogen acts for a longer period of time than two hours in this strain -- other experiments utilizing lower doses of 6-AN have shown that it does. In the C57BL strain a nicotinamide injection, following 6-AN administration, provided complete protection when given after one hour and partial protection when given after two (70%) or four (25%) hours, but no protection when given after six hours.

As was the case in the other experiments, the results tabulated in tables 9 and 10 can be interpreted in two ways -- either the C57BL strain has a larger nicotinamide pool, or the A/Jax strain
### Table 9

Number and weighted arc sine percentages of cleft palate

<table>
<thead>
<tr>
<th>Time of nicotinamide injection</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 AM</td>
<td>10</td>
<td>72</td>
<td>75</td>
<td>94.8%</td>
</tr>
<tr>
<td>9 AM</td>
<td>11</td>
<td>78</td>
<td>83</td>
<td>93.6%</td>
</tr>
<tr>
<td>11 AM</td>
<td>10</td>
<td>24</td>
<td>68</td>
<td>35.4%</td>
</tr>
<tr>
<td>1 PM</td>
<td>15</td>
<td>8</td>
<td>112</td>
<td>7.0%</td>
</tr>
<tr>
<td>2 PM</td>
<td>13</td>
<td>43</td>
<td>82</td>
<td>48.0%</td>
</tr>
<tr>
<td>3 PM</td>
<td>15</td>
<td>105</td>
<td>114</td>
<td>92.2%</td>
</tr>
<tr>
<td>5 PM</td>
<td>14</td>
<td>101</td>
<td>107</td>
<td>93.8%</td>
</tr>
<tr>
<td>7 PM</td>
<td>13</td>
<td>101</td>
<td>103</td>
<td>95.9%</td>
</tr>
<tr>
<td>no nicotinamide or control</td>
<td>10</td>
<td>76</td>
<td>77</td>
<td>96.1%</td>
</tr>
</tbody>
</table>

A/Jax embryos treated with a 3/4 dose of 6-AN on day 131/2 and protected with a critical dose of nicotinamide at various times before and after injection of 6-AN.
<table>
<thead>
<tr>
<th>Time of nicotinamide injection</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% cleft palate (weighted arc sine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 AM</td>
<td>10</td>
<td>26</td>
<td>84</td>
<td>32.5%</td>
</tr>
<tr>
<td>7 AM</td>
<td>10</td>
<td>25</td>
<td>80</td>
<td>29.3%</td>
</tr>
<tr>
<td>9 AM</td>
<td>12</td>
<td>24</td>
<td>95</td>
<td>21.2%</td>
</tr>
<tr>
<td>11 AM</td>
<td>12</td>
<td>7</td>
<td>90</td>
<td>7.7%</td>
</tr>
<tr>
<td>1 PM</td>
<td>11</td>
<td>0</td>
<td>84</td>
<td>3.2%</td>
</tr>
<tr>
<td>2 PM</td>
<td>11</td>
<td>3</td>
<td>79</td>
<td>5.7%</td>
</tr>
<tr>
<td>3 PM</td>
<td>15</td>
<td>24</td>
<td>116</td>
<td>18.8%</td>
</tr>
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<td>5 PM</td>
<td>14</td>
<td>50</td>
<td>104</td>
<td>47.4%</td>
</tr>
<tr>
<td>7 PM</td>
<td>14</td>
<td>67</td>
<td>100</td>
<td>68.6%</td>
</tr>
<tr>
<td>no nicotinamide or control</td>
<td>10</td>
<td>57</td>
<td>86</td>
<td>68.5%</td>
</tr>
</tbody>
</table>

Table 10
Number and weighted arc sine percentages of cleft palate C57BL embryos treated with a 3/4 dose of 6-AN on day 13½ and protected with a critical dose of nicotinamide at various times before and after injection of 6-AN.
has a greater affinity for 6-AN. Both explanations fit the data equally well and there is, again, no way of deciding which is correct. However, a third possible explanation was suggested by the series of experiments in which nicotinamide was injected prior to the administration of 6-AN. When nicotinamide was given eight hours before 6-AN in the C57BL strain it provided about 50% protection, but in the A/Jax strain it afforded no protection if given more than two hours prior to 6-AN. This implies that the C57BL strain can use exogenous nicotinamide more efficiently than the A/Jax strain, since it seems to be retained in the system for a longer time. A nicotinamide injection given four hours after administration of 6-AN was still able to provide protection (25%) in the C57BL strain, but not in the A/Jax strain. This also suggests that nicotinamide is retained longer in the C57BL strain than in the A/Jax strain. An alternate hypothesis is that nicotinamide is incorporated into NAD faster in the C57BL strain, but the results of the experiment with prior injection of nicotinamide favor the first explanation.

C. Cytoplasmic factor influencing the embryo's response to 6-AN:

Goldstein et al. (1963) found that the A/Jax
(A) strain had a higher frequency of cleft palate (76\%) than the C57BL (C) strain (11\%) after an intramuscular injection of 6-AN (19 mg/kg) on day 13^{1/2}, followed two hours later by an injection of nicotinamide (7.3 mg/kg). In addition to this strain difference, they reported a matroclinous reciprocal cross difference in the F_1 hybrids. Embryos from A mothers crossed to C fathers had a higher incidence of 6-AN-induced cleft palate (36\%) than the genetically identical (with the exception of the Y chromosome) embryos from C mothers crossed to A fathers (4\%). This maternal effect indicated that the mother's genotype was as important in determining the embryo's susceptibility to 6-AN-induced cleft palate as the embryo's genotype.

Two types of F_1 mothers (AC and CA) were backcrossed to the A strain and treated with 6-AN in an attempt to determine whether the maternal effect was due to uterine environment or "factors" transmitted through the egg cytoplasm -- the maternal and fetal genotypes were identical, but maternal cytoplasmic factors were different. AC x A embryos were significantly more susceptible to cleft palate (24\%) than CA x A embryos (6\%). Since the only difference between these crosses was the origin of the maternal cytoplasm, it was suggested that there were factors
in the A cytoplasm which increased the embryo's susceptibility to the teratogenic action of 6-AN on day 13\(\frac{1}{2}\) of gestation.

An alternate, but extremely unlikely, interpretation of the results is that the uterine environment provided by the mother is influenced by the uterine environment in which she, herself, developed (Goldstein et al., 1963). The backcrosses necessary to investigate this hypothesis have never been reported.

When, in the course of another experiment, the AC x A and CA x A crosses were repeated, the matroclinous reciprocal cross difference described above had vanished. The entire experiment was run a second time to clarify the situation. This time, however, intraperitoneal injections were used in an attempt to eliminate the possibility of leakage. The cleft palate frequency in the AC x A cross (43\%) did not show the expected increase above the CA x A frequency (46\%) (Humphreys, Rosenbaum, and Fraser, unpublished). These results were puzzling until it was noticed that the diet, which had consisted of Purine Lab Chow and water ad libitum (supplemented once a week with whole wheat bread, milk, and lettuce), had been changed shortly after completion
of the first experiment. Since the mice used in the second experiment had been maintained on Purina Mouse Breeder Chow, the chemical composition of the two diets was compared (Purina Laboratory Manual). The diets differed considerably in fat, protein, and mineral content, but these modifications in content most likely had little or no connection with factors involved in this experiment. The important difference between the diets, as far as this study was concerned, was probably the change in niacin content. Lab Chow contains 98.34 ppm of niacin, while Mouse Breeder Chow has only 53.33 ppm -- the tryptophan content of both diets was the same.

When the crosses were repeated a third time (Verrusio, unpublished) with mice that had been maintained on the original diet (Lab Chow), the cleft palate frequency for the AC x A cross (45%) was significantly higher (P 0.001) than that for the CA x A cross (26%) (see table 11 and figure 8).

An interesting observation emerges from a careful comparison of the "Mouse Breeder Chow" experiment (see figure 9). The cleft palate frequency is approximately the same for the AC x A cross on either Mouse Breeder Chow (43%) or Lab Chow (45%).

However, the CA x A cross has a cleft
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cross</th>
<th>Diet</th>
<th>Number of females treated</th>
<th>Number of cleft palate embryos</th>
<th>Total number of embryos</th>
<th>% Cleft palate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verrusio 66</td>
<td>AC x A</td>
<td>Purina Lab Chow</td>
<td>23</td>
<td>89</td>
<td>200</td>
<td>44.5</td>
</tr>
<tr>
<td></td>
<td>CA x A</td>
<td></td>
<td>30</td>
<td>76</td>
<td>295</td>
<td>25.8</td>
</tr>
<tr>
<td>Humphreys &amp; Rosenbaum 65</td>
<td>AC x A</td>
<td>Purina Mouse Breeder Chow</td>
<td>16</td>
<td>57</td>
<td>133</td>
<td>42.9</td>
</tr>
<tr>
<td></td>
<td>CA x A</td>
<td></td>
<td>18</td>
<td>72</td>
<td>157</td>
<td>45.9</td>
</tr>
<tr>
<td>Goldstein</td>
<td>AC x A</td>
<td>Purina Lab Chow</td>
<td>9</td>
<td>18</td>
<td>58</td>
<td>23.7</td>
</tr>
<tr>
<td></td>
<td>CA x A</td>
<td></td>
<td>11</td>
<td>4</td>
<td>69</td>
<td>5.5</td>
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</table>

Table 11
The influence of diet on reciprocal cross differences in cleft palate frequency following maternal treatment with 6-AN on day 13 1/2 of gestation.
Figure 8

The influence of diet on reciprocal cross differences in cleft palate frequencies following treatment with 6-AN on day 13 1/2 of gestation.
- Mouse Breeder Chow
  53.33 ppm Niacin

- Lab Chow
  98.43 ppm Niacin

% CP

Cross

<table>
<thead>
<tr>
<th>AC × A</th>
<th>CA × A</th>
<th>AC × A</th>
<th>CA × A</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOLDSTEIN/63</td>
<td>HUMPHREY + ROSENBAUM/64</td>
<td>VERRUSIO/65</td>
<td></td>
</tr>
<tr>
<td>Diet</td>
<td>AC x A (A Jax cytoplasm and mitochondria)</td>
<td>CA x A (C57BL cytoplasm and mitochondria)</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------</td>
<td>-------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>MOUSE BREEDER CHOW</td>
<td>1 Dose 6 AN 1 Dose N 43 % CP 53.33 ppm Niacin (in diet)</td>
<td>1 Dose 6 AN 1 Dose N 46 % CP 53.33 ppm Niacin (in diet)</td>
<td></td>
</tr>
<tr>
<td>LAB CHOW</td>
<td>1 Dose 6 AN 1 Dose N 45 % CP 98.34 ppm Niacin (in diet)</td>
<td>1 Dose 6 AN 1 Dose N 26 % CP 98.34 ppm Niacin (in diet)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 9
palate frequency of 45% on Mouse Breeder Chow and only 26% on Lab Chow. Why should CA x A embryos have a different cleft palate frequency on the two diets, and why should embryos from AC x A and CA x A crosses have a different cleft palate frequency on the Lab Chow diet, when they have the same genotype, (AC)A and (CA)A, and come from mothers with the same genotype, AC and CA? As stated before, the one important difference between the two diets is that Lab Chow has twice as much niacin as Breeder Chow. The only difference between the embryos from the two crosses is that the cytoplasm of AC mothers is derived from the A strain and that of CA mothers is derived from the C strain. It would seem, then, that the embryos with C cytoplasm can use exogenous nicotinamide more efficiently, at some concentrations, than those with A cytoplasm. When the mice were maintained on Mouse Breeder Chow, the cleft palate frequency following treatment with 6-AN was approximately the same in both crosses. This suggests that the amount of niacin present in Mouse Breeder Chow must be close to the animals' actual requirement for the vitamin, since C cytoplasm was not able to help forestall the action of 6-AN any better than A cytoplasm. On the other hand, when the mice were maintained on Lab Chow, which has twice
as much niacin, C cytoplasm helped lower the cleft palate frequency by one-half.

The factor in the cytoplasm which is responsible for this matroclinous reciprocal cross difference cannot be determined by nuclear genes, because the AC and CA genomes theoretically contribute the same materials to the cytoplasm. The only organelles in the cytoplasm of mouse eggs that are self-duplicating and do not arise de novo are the mitochondria. The DNA of these organelles represents a multigenic hereditary system which is not derived from the nucleus, and which is responsible, in part, for the specific biochemical properties of the organelle (Gibor and Granick, 1964). Variations in the genetic properties of different mitochondrial-gene mutants have been observed in yeast. Ephrussi (1953) and Ephrussi et al. (1955) found two types of respiratory-deficient cells, suppressive and neutral. When neutral respiratory-deficient cells were crossed with cells containing normal mitochondria the progeny had normal mitochondria, but strains derived from crosses between suppressive respiratory-deficient cells and normal cells had inhibited respiration. The suppressive cells could also mutate to the neutral state.

Caspari and Santway (1954) and Caspari
(1956) demonstrated differences between the mitochondria of different strains of mice. They found that liver mitochondria from C57BL males differ from those of BALB males by having a higher phosphorous/nitrogen ratio, and that this difference is at least in part due to a difference in the RNA/protein ratio of that organelle. The results of crosses between the two strains were interpreted in terms of an interaction of a cytoplasmic factor with nuclear genes.

In animals some instances have been found in which mitochondria are inherited only through the egg, not through the sperm. Bourne (1951) has cited a number of examples, among them the following. In Nereis the middle piece of the sperm which carries the mitochondrial material does not enter the egg at all. The individual in this case contains the chromosomes of both parents, but only the mother's mitochondria. When the fertilized egg of the bat divides, the male mitochondria sometimes pass into only one of the first two cells. And in echinids, the male mitochondria can be traced into only one cell of a 32-cell embryo. This is not to imply that the situation in mice is the same. In fact, Goldstein (unpublished) had some evidence for a reciprocal cross difference in cleft palate frequency when CA and AC males were
et al., 1954) leads to the synthesis of NAD analogs containing 6-AN instead of nicotinamide (Dietrich et al., 1958), must take place inside the mitochondria. Thus, 6-AN and nicotinamide must be able to cross the mitochondrial membrane in both A and C mitochondria. The possibility that a differential permeability for 6-AN exists between A and C mitochondria is ruled out by the results of the Mouse Breeder Chow experiment. This same experiment also eliminates the possibility that the A and C mitochondrial enzymes which catalyze the exchange reactions (NADases) have differential affinities for 6-AN, since the cleft palate frequencies of the reciprocal crosses are almost the same (43% and 46%). The fact that the same experimental procedure elicits a reciprocal cross difference (table 11) when the diet contains more niacin (Lab Chow) indicates that the way the animal handles nicotinamide is responsible for the strain difference in response to the teratogen 6-AN.

When the mice were maintained on Lab Chow, the cleft palate frequency of AC x A embryos was 45%, while that of CA x A embryos was only 26%. These results are interesting in that they suggest that C mitochondria have the ability to "store" excess nicotinamide (as NAD?) and, as demonstrated in the
mated to A/Jax females. This could result from a situation somewhat like that postulated by Gibor and Granick (1964) in which both types of mitochondria would be carried along in the hybrid animal until some drastic environmental change occurred. Then there would be selection for the most suitable organelles. A diet with a high niacin content can hardly be described as a drastic environmental change, but it could impart a selective advantage to mitochondria which are able to use niacin more efficiently.

The cytoplasmic factor experiment is currently being repeated a fourth time. This time embryos reared on Mouse Breeder Chow, supplemented with niacin, will also be included to test if the important difference between the two diets is really the niacin content. The paternal effect, which disappeared on Lab Chow (Humphreys, Rosenbaum, and Fraser, unpublished), will be tested again.

The antimetabolite 6-AN has been shown to interfere with oxidative phosphorylation in liver mitochondria by forming an NAD analog (Nanni et al., 1961). Since external (cytoplasmic) pyridine nucleotides penetrate the intact mitochondria only with great difficulty (White et al., 1964), the exchange reaction, which under the influence of NADase (Kaplan
kinetic study, retain it in the system longer than A mitochondria. This leads to the hypothesis that there is a genetically controlled difference in the efficiency with which the A/Jax and C57BL strains handle nicotinamide, resulting in a difference in the size of their intra-mitochondrial pools of NAD.

The results of the kinetics studies of 6-AN teratogenesis reported above all seem to support this hypothesis. The alternate hypotheses of differential enzyme affinities and coenzyme turnover rates have been discarded along the way as evidence was presented against them.

D. 6-AN treatment and amniotic fluid volume:

Harris (1964) demonstrated that cortisone treatment of mice reduces amniotic fluid volumes, and postulated that constriction of the embryo resulting from this decrease in fluid was the cause of cortisone-induced cleft palate, rather than interference with acid-mucopolysaccharide synthesis (Walker and Fraser, 1957; Larsson, 1962).

Walker (1965) corroborated the reduction in amniotic fluid volume after treatment with a dose of cortisone that produced 100% cleft palate, but felt that it was not primarily responsible for the induced cleft palates, because some control embryos
had as little fluid as treated embryos, and some treated embryos had as much fluid as controls. However, in a multifactorial threshold system, this observation in not unequivocal proof that the hypothesis is incorrect. A critical test of the hypothesis would be a comparison of amniotic fluid volumes in embryos with and without cleft palate in litters from mothers treated with a dose of cortisone which causes 50% cleft palate (Fraser, Chew and Verrusio, in press).

To see whether 6-AN also reduces amniotic fluid volume, pregnant A/Jax mice were injected, intraperitoneally, with a $\frac{1}{2}$ dose of 6-AN on day $13\frac{1}{2}$ of gestation. This dose of 6-AN produced cleft palates in 46% of the surviving embryos, however, it did not significantly reduce the amount of amniotic fluid below the control value. Nor was the amniotic fluid volume significantly less in embryos with cleft palate than in their normal litter-mates (table 12).

The mean weight of the cleft palate embryos was significantly lower than that of their normal litter-mates. This suggests that 6-AN does not affect all the embryos in a litter to the same extent. Whether cleft palate embryos are affected because they are small, or small because they are affected, is something
<table>
<thead>
<tr>
<th>Cross</th>
<th>A/Jax x A/Jax</th>
<th>6-AN</th>
<th>6-AN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Control</td>
<td>Normal</td>
<td>Cleft</td>
</tr>
<tr>
<td>Palate</td>
<td>Normal</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Number of females</td>
<td>37</td>
<td>42</td>
<td>49</td>
</tr>
<tr>
<td>Mean wt. of embryos</td>
<td>428.3</td>
<td>378.4</td>
<td>433.1</td>
</tr>
<tr>
<td>Standard error</td>
<td>6.6</td>
<td>8.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Amniotic fl. wt.</td>
<td>167.9</td>
<td>155.5</td>
<td>162.0</td>
</tr>
<tr>
<td>Standard error</td>
<td>2.8</td>
<td>2.7</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Table 12
Weight of embryos and amniotic fluid in embryos with or without cleft palate following treatment with 6-AN.
that can only be resolved by using radioactive tracer techniques. The weight loss of treated cleft palate embryos is not enough, in itself, to cause cleft palate. Chamberlain and Nelson (1963b) found that severe fasting of pregnant rats, which more than simulated the weight loss following 6-AN treatment, did not result in cleft palate production.
V. SUMMARY

The critical period for cleft palate production in both the A/Jax and C57BL strains following a two hour inactivation of nicotinamide by 6-aminonicotinamide at half-day intervals was found to be day 13½. This extended and confirmed the findings of Goldstein et al. (1963). Although palate closure commences half a day earlier in C57BL mice than in A/Jax mice, the period of maximum sensitivity of the palate to this teratogen is the same in both strains. Since the C57BL strain required more 6-AN than the A/Jax strain to produce a comparable cleft palate frequency on day 13½, it may correctly be termed a "resistant" strain.

The dose-response patterns of both the A/Jax and C57BL strains appear to be sigmoid. A probit transformation was used to test the sigmoidicity of the curves. When the probit transformations were plotted against dose, a simple regression analysis showed a good linear correlation for both strains, suggesting that the dose-response curves of the original data were, indeed, sigmoid. The only other conclusion one can draw from this experiment is that
it takes more 6-AN to cause cleft palate in the C57BL strain than in the A/Jax strain.

The equivalent, or critical, dose of nicotinamide -- that amount which exactly counteracts the teratogenic effects of a given amount of 6-aminonicotinamide -- was found for the A/Jax and C57BL strains. The results indicate that the C57BL strain needs less nicotinamide than the A/Jax strain (3.19 mg/kg vs 3.42 mg/kg) to counteract a larger dose of 6-aminonicotinamide (16.63 mg/kg vs 11.88 mg/kg). If the ratio of nicotinamide to 6-aminonicotinamide is about 1 to 3.5 in the A/Jax strain, but only 1 to 5.2 in the C57BL strain, then obviously the A/Jax strain requires one and a half times as much nicotinamide as the C57BL strain to counteract 6-aminonicotinamide at these dose levels.

When an amount of nicotinamide equivalent in each strain to a $\frac{3}{4}$ dose of 6-aminonicotinamide was injected into pregnant females at times varying from 0 to 8 hours before or after administration of a $\frac{3}{4}$ dose of 6-aminonicotinamide the following observations were made: a dose of nicotinamide given 8 hours before injection of 6-AN still affords about 50% protection in the C57BL strain, while in A/Jax mice the ability of a prior injection to forestall
cleft palate production by 6-aminonicotinamide is almost completely lost if it is given four hours before the teratogen; simultaneous injection of nicotinamide and 6-aminonicotinamide provided almost complete protection in both strains, as has been previously reported (Pinsky and Fraser, 1960; Goldstein, 1964); 6-aminonicotinamide is effective in causing developmental damage for at least four hours in the C57BL strain. (A 3/4 dose of 6-aminonicotinamide causes 100% cleft palate in the A/Jax strain after a two hour exposure, so nothing can be said about the effective period of 6-aminonicotinamide in this strain.) The results suggest that nicotinamide is retained longer in the C57BL strain than in the A/Jax strain.

A matroclinous reciprocal cross difference in cleft palate frequency was found when the AC x A (45%) and CA x A (26%) crosses were done with mice that had been maintained on Lab Chow (high niacin content). These results are interesting in that they suggest that C57BL "cytoplasm" (mitochondria) have the ability to "store" excess nicotinamide (as NAD perhaps), and retain it in the system longer than A/Jax cytoplasm (mitochondria). This leads to the hypothesis that there is a genetically controlled difference
in the efficiency with which the A/Jax and C57BL strains handle nicotinamide, resulting in a difference in the size of their intra-mitochondrial pools of NAD.

A 1/2 dose of 6-aminonicotinamide produced cleft palates in 46% of the surviving embryos, however, it did not reduce the amniotic fluid volume below the control value. Nor was the amniotic fluid volume significantly less in cleft palate embryos than in their normal litter-mates. The mean weight of cleft palate embryos was significantly lower than that of their normal litter-mates which weighed the same as the controls. This suggests that 6-aminonicotinamide does not affect all the embryos in a litter to the same extent.
VI. ACKNOWLEDGMENTS

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