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Antimetabolites in the Nutrition of *Aedes aegypti* L. Larvae

NICOTINIC ACID ANALOGUES

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(Received 28 December 1962)

Nicotinic acid antagonists have been employed in studies of insect nutrition (Shyamala & Bhat, 1958; Levinson & Bergmann, 1959), insect tissue cultures (Sanborn & Haskell, 1960), and insect development (Blaustein & Schneiderman, 1960). The effects obtained have usually been attributed to vitamin deficiencies. However, there is little direct evidence that antimetabolites affect insects in the same manner as they do higher animals. Few quantitative studies on the antivitamin-vitamin relationship, with insects as experimental animals, are available. The purpose of the present study was to investigate the effect of five better-known nicotinic acid analogues on *Aedes aegypti* L. larvae, grown on a chemically defined diet.

The specificity of the nicotinic acid requirement of various organisms has been extensively studied. Dietary nicotinic acid can be replaced by tryptophan, many nicotinamide derivatives, and even some nicotinic acid analogues (Hundley, 1954). Small doses of 3-acetylpyridine had a vitamin-like activity for the dog and the rat (McDaniel, Hundley & Sebrell, 1955; Guggenheim & Diamant, 1958). The nicotinamide-3-acetylpyridine relationship was, therefore, studied in mosquito larvae, given a basic casein diet, on which the exact nicotinamide requirements were determined.

MATERIALS AND METHODS

Methods of obtaining sterile larvae, of preparing the basal media, of testing their sterility, and of evaluating the results were described by Akov (1962). One larva was reared per tube, and its stage recorded daily until it

reached the adult stage or died. The results are expressed by an index, consisting of the sum of percentages of pupae and of adults divided by the median day of pupation. An index of about 25 was usually obtained on the basic diet (at 28°), and can be considered to be the optimum. Under these conditions up to 100% of the larvae reached the adult stage with a median larval period of 8 days. An index of zero is considered to indicate complete inhibition. The inhibition index is the ratio of moles of antimetabolite to moles of vitamin that causes 50% inhibition (Woolley, 1952).

The basal nicotinamide-free diet consisted of solid vitamin-free casein (50 mg./tube) and 5 ml. of liquid medium of the following composition: RNA (sodium salt), 1 mg./ml.; inorganic salts, 1.45 mg./ml.; cholesterol, 6 µg./ml.; biotin, 0.1 µg./ml.; folic acid, 0.6 µg./ml.; thiamine hydrochloride, 2 µg./ml.; riboflavin, 2 µg./ml.; pyridoxine hydrochloride, 4 µg./ml.; calcium pantothenate, 10 µg./mg.; choline chloride, 100 µg./ml. The appropriate amount of nicotinamide and the analogue were added before the larva was inoculated.

RESULTS

The effect of graded doses of nicotinic acid and nicotinamide is given in Table 1. Both forms of the vitamin were equally effective. The effect of various antimetabolites was tested on a diet containing the minimal amount of nicotinamide (2 µg./ml.) to give optimum results. Any possible sparing effect was tested in the presence of 0.5–2.0 µg. of nicotinamide/ml. Amounts of nicotinamide up to 2 mg./ml. had no detrimental effect.

The addition of L-tryptophan to nicotinamide-free or low-nicotinamide diets had no beneficial effect (Table 2).

The toxic effect of five nicotinic acid analogues, when added to a low-nicotinamide diet, was tested, and their respective inhibition indices were computed (Table 3). The toxicity of 3-acetylpyridine and α -picolinic acid for mosquito larvae was several times higher than that of pyridine-3-sulphonic acid, 6-aminonicotinamide and isonicotinic acid hydrazide. [The last-named, which is a pyridoxine rather than a nicotinic acid antagonist (Rosen, 1955), was tested on a medium also containing a minimal amount of pyridoxine]. In the presence of 2 and 4 mg./ml. of pyridine-3-sulphonic acid, a reddish precipitate was formed on the bottom of the tube. This effect might have been caused by the chelating property of pyridine-3-sulphonic acid, but this aspect was not investigated further.

The nicotinamide-sparing activity of the nicotinic acid analogues was tested by the addition of a single non-toxic dose of each analogue to nicotinamide-free or low-nicotinamide diets. 3-Acetylpyridine was the only compound that had any growth-promoting activity (Table 4).

The nicotinamide-sparing effect of small amounts of 3-acetylpyridine has been investigated quantitatively (Table 5). Small doses of 3-acetylpyridine could completely replace nicotinamide in the nicotinamide-free diet, and had a nicotinamide-sparing activity on diets with sub-optimum amounts of nicotinamide. The activity of 3-acetylpyridine was about one-tenth of that of nicotinamide. The mosquitoes on diets containing only 3-acetylpyridine were active and looked normal.

Table 1. *Effect of graded doses of nicotinic acid or nicotinamide on a nicotinic acid-free basic diet*

Experimental details are given in the text.

$$\text{Index} = \frac{\% \text{ of pupae} + \% \text{ of adults}}{\text{median day of pupation}}.$$

Addition	Amount ($\mu\text{g./ml.}$)	No. of larvae	No. of pupae	No. of adults	Median day of pupation	Index
None	—	85	0	—	—	0
Nicotinic acid	0.02	12	0	—	—	0
	0.2	10	0	—	—	0
	2	10	9	9	7.5	24.0
	10	9	9	9	8.5	23.5
	20	10	10	9	8	23.8
	200	9	9	9	9.5	20.1
Nicotinamide	0.02	11	0	—	—	0
	0.2	36	1	1	20	0.3
	0.5	66	26	19	19	5.3
	1	59	51	45	10.5	15.5
	2*	139	136	126	8.5	22.2
	10†	77	76	72	8.5	22.6
	20	24	23	22	8.5	22.1
	200	56	56	48	8.5	21.2

* Amount of nicotinamide used for testing of antimetabolites.

† Amount of nicotinamide in standard basic diet.

Table 2. *Inability of L-tryptophan to act as substitute for nicotinamide in Aedes aegypti larvae*

Experimental details are given in the text.

$$\text{Index} = \frac{\% \text{ of pupae} + \% \text{ of adults}}{\text{median day of pupation}}.$$

Amount of nicotinamide ($\mu\text{g./ml.}$)	Addition	No. of larvae	No. of pupae	No. of adults	Median day of pupation	Index
0	None	10	0	0	—	0
0.5		10	6	4	33	3.0
1		10	7	6	12	10.8
2		10	10	10	8.5	23.5
10		12	12	11	8	24
0	Tryptophan (2 mg./ml.)	11	0	0	—	0
0.5		10	1	0	33	0.3
1		11	4	4	21	3.5
2		10	9	9	10	18.0
10		9	9	9	9	22.2

The vitamin-like activity of 3-acetylpyridine, which was rather toxic in comparison with other nicotinic acid analogues, led us to investigate the effect of graded doses of this compound with different amounts of dietary nicotinamide. The

toxicity of 3-acetylpyridine was independent of the amount of nicotinamide in the diet (Table 6). With the nicotinamide-free diet and with 0.5 µg. of nicotinamide/ml., the growth-promoting effect of 3-acetylpyridine rose until it reached the maxi-

Table 3. *Relative toxicities of nicotinic acid analogues to Aedes aegypti larvae fed on a diet containing a minimal amount of nicotinamide*

The diet contained 2 µg. of nicotinamide (mol.wt. 122.12)/ml. Experimental details are given in the text.

$$\text{Index} = \frac{\% \text{ of pupae} + \% \text{ of adults}}{\text{median day of pupation}}$$

$$\text{Inhibition index} = \frac{\text{moles of inhibitor}}{\text{moles of metabolite}} \text{ to cause 50\% inhibition.}$$

Analogue added	Amount (mg./ml.)	No. of larvae	No. of pupae	No. of adults	Median day of pupation	Index	Inhibition index
α -Picolinic acid hydrochloride (mol.wt. 159.6)	0	35	34	31	9.5	19.6	~ 60
	0.1	24	20	20	11	15.2	
	0.2	31	15	13	12	7.5	
	0.5	20	8	8	12.5	6.4	
	1.0	10	0	—	—	0	
3-Acetylpyridine (mol.wt. 121.13)	0	9	9	9	8.5	23.5	~ 100
	0.1	23	23	23	9	22.2	
	0.2	22	15	10	11.5	9.9	
	0.5	19	0	—	—	0	
Isonicotinic acid hydrazide (mol.wt. 137.12)	0	21	20	20	8	23.8	~ 350
	0.1	20	19	18	8.5	21.8	
	0.2	20	19	18	9	20.6	
	0.5	19	15	15	10.5	15.0	
	1	20	10	10	12.5	8.0	
Pyridine-3-sulphonic acid (mol.wt. 159.16)	2	21	0	—	—	0	~ 400
	0	16	16	16	8.5	23.5	
	0.1	10	10	10	10	20.0	
	0.2	17	17	14	10	18.2	
	0.5	16	16	16	9.5	21.1	
6-Aminonicotinamide (mol.wt. 137.12)	1	17	13	12	12.5	11.8	~ 500
	2	17	6	6	15	4.7	
	4	9	0	—	—	0	
	0	21	20	17	8.5	20.7	
	0.1	21	20	20	9	21.2	
	0.2	19	15	13	10	14.7	
	0.5	15	13	11	11	14.5	
	1	18	17	13	13.5	12.3	
	1.5	10	5	3	15	5.3	

Table 4. *Effect of nicotinic acid analogues added to nicotinamide-free and low-nicotinamide diets given to Aedes aegypti larvae*

Experimental details are given in the text.

$$\text{Index} = \frac{\% \text{ of pupae} + \% \text{ of adults}}{\text{median day of pupation}}$$

The numbers in parentheses refer to the numbers of larvae.

Analogue added	Amount of nicotinamide (µg./ml.)			
	0	0.5	1.0	2.0
	Index			
None	0(39)	3.6 (40)	17.3 (39)	21.3 (32)
3-Acetylpyridine (50 µg./ml.)	25.0 (5)	25.0 (6)	—	22.9 (18)
Pyridine-3-sulphonic acid (50 µg./ml.)	0 (13)	2.4 (11)	14.4 (12)	25.5 (11)
Isonicotinic acid hydrazide (50 µg./ml.)	0 (13)	4.5 (13)	17.0 (13)	22.2 (12)
6-Aminonicotinamide (10 µg./ml.)	0 (18)	0.8 (18)	12.1 (18)	20.3 (11)
α -Picolinic acid (10 µg./ml.)	0 (12)	4.7 (12)	19.0 (9)	18.2 (11)

Table 5. *Nicotinic acid-sparing effect of 3-acetylpyridine in Aedes aegypti larvae*

Experimental details are given in the text.

$$\text{Index} = \frac{\% \text{ of pupae} + \% \text{ of adults}}{\text{median day of pupation}}.$$

The numbers in parentheses refer to the numbers of larvae.

Amount of 3-acetyl- pyridine ($\mu\text{g./ml.}$)	Amount of nicotinamide ($\mu\text{g./ml.}$)				
	0	0.5	1.0	1.5	2.0
	Index				
0	0 (17)	3.5 (16)	16.7 (10)	23.7 (10)	21.3 (24)
5	4.5 (18)	11.5 (16)	23.5 (10)	23.7 (11)	25.0 (18)
10	11.8 (17)	16.0 (16)	21.2 (10)	23.8 (10)	20.3 (18)
20	20.0 (7)	23.7 (5)	—	—	26.7 (10)

Table 6. *Effect of graded doses of 3-acetylpyridine with different amounts of nicotinamide in the diet given to Aedes aegypti larvae*

Experimental details are given in the text.

$$\text{Index} = \frac{\% \text{ of pupae} + \% \text{ of adults}}{\text{median day of pupation}}.$$

The numbers in parentheses refer to the numbers of larvae.

Amount of 3-acetyl- pyridine ($\mu\text{g./ml.}$)	Amount of nicotinamide ($\mu\text{g./ml.}$)				
	0	0.5	2	20	200
	Index				
0	0 (17)	3.5 (16)	21.3 (24)	23.5 (5)	26.7 (5)
100	23.5 (5)	22.9 (7)	22.2 (23)	21.3 (10)	23.7 (9)
150	23.5 (8)	21.1 (7)	20.2 (11)	18.5 (12)	20.0 (10)
200	17.8 (5)	21.1 (6)	10.9 (11)	22.2 (10)	16.8 (10)
250	15.0 (6)	10.8 (5)	8.7 (22)	9.7 (12)	8.3 (11)
300	1.5 (5)	5.5 (6)	7.4 (7)	0 (7)	5.5 (6)
400	0 (6)	0 (5)	0 (5)	0 (5)	0 (5)

num. With 200 $\mu\text{g.}$ of the analogue/ml. the growth-promoting effect was gradually cancelled by the toxic effect, and with 250 $\mu\text{g./ml.}$ it caused about 50 % inhibition as measured by the index. About half of the larvae pupated and most pupae emerged. Development was slower than under optimum conditions (the median time of pupation was 11–12 days). With 300 $\mu\text{g.}$ of 3-acetylpyridine/ml., less than half of the larvae pupated and became adults; development was very slow. In the presence of 400 $\mu\text{g.}$ of 3-acetylpyridine/ml. the larvae reached only the second or third instar. With 500 $\mu\text{g./ml.}$ all larvae died in the first instar within a day or two. These manifestations of toxicity were the same with all amounts of dietary nicotinamide investigated. The concentrations at which 3-acetylpyridine was inhibitory were about ten times as great as those required to manifest its vitamin-like activity. Five times the minimal amount of 3-acetylpyridine required for optimum growth was well tolerated with all amounts of nicotinamide in the diet.

Finally, the ability of nicotinamide to overcome

the inhibitory effect of α -picolinic acid, pyridine-3-sulphonic acid and 6-aminonicotinamide was tested, with diets that contained 10 and 100 times the amount of nicotinamide required. The toxic effect of these nicotinic acid antagonists was also independent of the amount of vitamin in the medium.

DISCUSSION

The fact that tryptophan could not replace nicotinamide for mosquito larvae was not unexpected. Tryptophan did not replace nicotinic acid in other insects (Lipke & Fraenkel, 1956). The yellow pigment excreted by rice-moth larvae on a pyridoxine-deficient diet, to which tryptophan was added, was found to be 3-hydroxykynurenine (Sundaram, Radhakrishnamurty, Shanmugasundaram & Sarma, 1953). All these experiments, however, were conducted in the presence of an adequate amount of nicotinic acid in the diet, and thus offer no evidence that 3-hydroxykynurenine is further converted into nicotinic acid. The total amount of nicotinic acid in the silkworm was

found to increase during the pupal stage, though tryptophan could not be identified as a precursor (Kato & Hamamura, 1952). The tryptophan-nicotinic acid relationship, as known from higher animals and *Neurospora*, has never been demonstrated in an insect.

α -Picolinic acid and 3-acetylpyridine were the most toxic analogues for mosquito larvae, whereas isonicotinic acid hydrazide and pyridine-3-sulphonic acid were tolerated in large doses. In this respect, the mosquito larvae, like other insects to which these nicotinic acid analogues have been applied (Shyamala & Bhat, 1958; Levinson & Bergmann, 1959; Blaustein & Schneiderman, 1960), resemble higher animals rather than micro-organisms (Woolley, 1952). The inability of nicotinamide to overcome the toxic effect of 3-acetylpyridine in mosquito larvae was also similar to that of other higher organisms and insects. The toxic effect of 3-acetylpyridine could not be reversed by nicotinic acid in the dog and the rat (McDaniel *et al.* 1955; Guggenheim & Diamant, 1958) but was reversible in mice and chick embryos (Woolley, 1952). The addition of nicotinic acid to silkworms treated with 3-acetylpyridine reduced their mortality, but the weight of the recovered worms did not reach 30 % of that of the controls (Shyamala & Bhat, 1958). The weight and survival of housefly larvae treated with 3-acetylpyridine was somewhat improved by nicotinic acid, but pupation was less than 10 % (Levinson & Bergmann, 1959). 6-Aminonicotinamide was the least toxic of all the analogues investigated. This compound, which is a potent nicotinamide antagonist in mammals, has not been tested in other insects. 6-Aminonicotinamide was ten times as toxic as 3-acetylpyridine for mice, and this effect was reversible by nicotinamide (Johnson & McColl, 1955).

The study of the nicotinic acid activity of various pyridine derivatives showed that, in order to retain biological activity for higher animals and micro-organisms, the pyridine molecule must retain a substituent group in position 3 (Hundley, 1954). This is true also for insects. α -Picolinic acid (2-pyridinecarboxylic acid) and isonicotinic acid (4-pyridinecarboxylic acid), which were inactive for mosquito larvae, had no nicotinic acid activity for other insects (Ellinger, Fraenkel & Abdel-Kader, 1947). Pyridine-3-sulphonic acid, which had some growth-promoting effect for micro-organisms (Hundley, 1954), had no nicotinamide activity for mosquito larvae. 6-Aminonicotinamide was also inactive. Though isonicotinic acid hydrazide and 6-aminonicotinamide had no vitamin-like activity in any organism, their pyridine nucleotide analogues have been prepared and isolated *in vitro* (Zatman, Kaplan, Colowick & Ciotti, 1954; Dietrich, Friedland & Kaplan, 1958).

In our experiments 3-acetylpyridine was the only pyridine derivative to have any growth-promoting activity for mosquito larvae. No difference between mosquitoes raised on 3-acetylpyridine alone and mosquitoes raised on the basic nicotinamide-containing diet could be detected by the criteria used in our work. The two-sided effect of 3-acetylpyridine, which acted both as a vitamin and an antivitamin for the dog and the rat, has been explained by the conversion of the analogue into nicotinic acid (McDaniel *et al.* 1955; Guggenheim & Diamant, 1958). When the dosage of 3-acetylpyridine exceeded the animal's capacity for detoxification, the excess of the analogue acted as an antagonist. The mosquito larva was able to convert 3-acetylpyridine into the vitamin in a similar way to that in mammals. The capacity of the mosquito for this conversion was, however, independent of the amount of dietary nicotinamide. The ability of the dog to detoxify 3-acetylpyridine was impaired when the animal was in a state of nicotinic acid deficiency (McDaniel *et al.* 1955).

SUMMARY

1. The effect of graded doses of five nicotinic acid analogues on *Aedes aegypti* larvae, grown on a chemically defined diet, was tested.

2. Nicotinic acid and nicotinamide were both equally effective as growth factors. Tryptophan could not replace or spare nicotinamide.

3. The inhibition indices of the analogues, obtained on diets containing minimum nicotinamide, were as follows: α -picolinic acid, 60; 3-acetylpyridine, 100; isonicotinic acid hydrazide, 350; pyridine-3-sulphonic acid, 400; 6-aminonicotinamide, 500. The toxic effect of the nicotinic acid antagonists could not be reversed by nicotinamide.

4. Small doses of 3-acetylpyridine could replace nicotinamide as a growth factor. The amount of 3-acetylpyridine required for maximal development was ten times that of nicotinamide. The other four analogues had no nicotinic acid-sparing activity.

5. The nicotinic acid metabolism of insects and the effect of nicotinic acid analogues, as compared with those of other organisms, have been discussed.

We thank Professor G. S. Fraenkel of the University of Illinois, Urbana, for his helpful discussions and comments on the typescript, and for his interest and encouragement in this study. The work was supported in part by a research grant from the Ford Foundation.

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The Oxidation of Various Precursors in Normal and Tumour-Bearing Mice *in vivo*

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(Received 17 December 1962)

As a preliminary to studies of the incorporation of various precursors into cholesterol in normal and tumour-bearing mice, the oxidative fate of these metabolites was studied by measuring expiratory $^{14}\text{CO}_2$. Although expired CO_2 reflects an important end product of ingested carbon, it is not specifically related to any single biochemical event. Despite this lack of specificity, such tests as measurements of basal metabolism and determinations of respiratory quotient, which depend in part on measurements of CO_2 , have in the past been of great value. These same measurements can now be made more important when the relative contribution of various carbon sources to expired CO_2 are determined with the use of ^{14}C -labelled compounds. It is possible that a progressively growing tumour may cause subtle changes in the normal excretion pattern of CO_2 shown by the host before clinical signs are evident. The present studies were initiated in order to determine whether changes caused by a tumour would be reflected by a change in the rate of appearance of ^{14}C -labelled metabolites in expired air. A preliminary report of our work has been presented (Kabara, Okita & LeRoy, 1957).

MATERIALS AND METHODS

Animals and tumours. Female CF-1 or DBA/2 mice (10–12 weeks of age) weighing 20–25 g. were used as normal controls. The CF-1 mice, inoculated intraperitoneally or subcutaneously with Ehrlich ascites carcinoma cells, were used for the initial respiratory studies. Because experiments with mice bearing ascites tumours were complicated by the added factor of a change in the surface area of the mouse, the major part of the study was carried out with mice that had a solid tumour mass. Similar experiments were also carried out with the DBA/2 mice, inoculated with a lymphatic leukaemia (P-1534). All mice were bred and raised at the Argonne Cancer Research Hospital.

Radioactive metabolites. The doses of ^{14}C -labelled compounds injected into mice were all considered to be trace quantities, i.e. the normal physiology of the animal was not upset by the amount of injected material. The following doses were given either intraperitoneally or intravenously in 0.5 ml. of 0.85% NaCl: $\text{NaH}^{14}\text{CO}_3$ (1.0 mc/m-mole), 0.05 μC ; $[1\text{-}^{14}\text{C}]\text{acetate}$ (2.8 mc/m-mole), 0.5 μC ; $[2\text{-}^{14}\text{C}]\text{acetate}$ (2.0 mc/m-mole), 1.0 μC ; $[2\text{-}^{14}\text{C}]\text{leucine}$ (0.6 mc/m-mole), 1.25 μC ; $[1\text{-}^{14}\text{C}]\text{glucose}$ (0.65 mc/m-mole), 1.0 μC ; $[6\text{-}^{14}\text{C}]\text{glucose}$ (0.83 mc/m-mole), 1.0 μC ; uniformly labelled $[^{14}\text{C}]\text{glucose}$ ($[\text{U-}^{14}\text{C}]\text{glucose}$) (1.00 mc/m-mole), 1.0 μC . All the radioactive compounds were purchased from Volk Radiochemical Co., 8260 Elmwood Avenue, Skokie, Ill., U.S.A.