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ON THE TOXIC ACTION OF TOXOPYRIMIDYL ACETYLPYRIDINE

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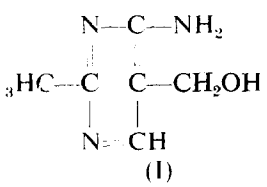
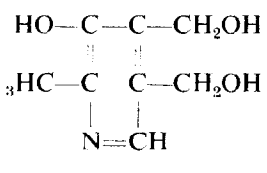
Abstract—Toxopyrimidyl acetylpyridine (TXP-AcP) was synthesized by condensing 2-methyl-4-amino-5-bromomethylpyrimidine dihydro-bromide with 3-acetylpyridine. This is a toxic compound which differs completely in its pharmacological properties from its components, toxopyrimidine (TXP) or 3-acetylpyridine (3-AcP). Though on injection TXP-AcP presents similar symptoms to KCN in mice, in enzymatic experiments the sites of action of both poisons differ from each other.

Phenobarbital and veronal can protect the animals against the toxicity of TXP-AcP, and by artificial respiration, TXP-AcP-injected mice are relieved. TXP-AcP appears to act directly on the respiratory centre.

IT IS well known that mice treated with toxopyrimidine[1] (TXP) (I), the pyrimidine moiety* of vitamin B_6, exhibit running fits and die about 2 hr after the injection, and that B_6-vitamins (II) can protect the animals against the toxicity of TXP.

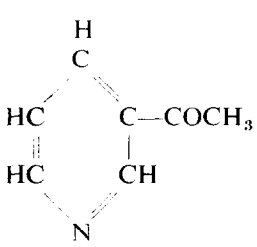


(I)

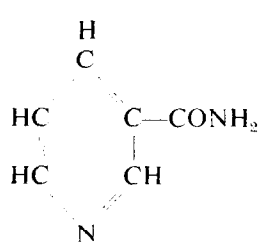


(II)

Pyridoxine (—CHO) Pyridoxal (—CH_2NH_2) Pyridoxamine

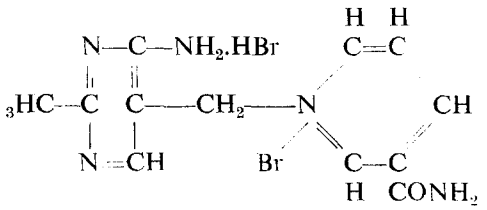


(III)



(IV)

* 2-Methyl-4-amino-5-hydroxymethylpyrimidine.



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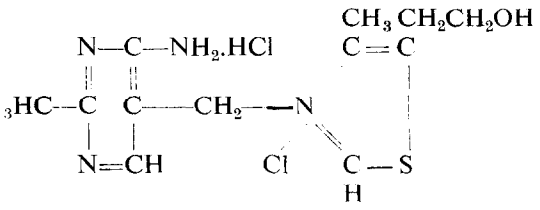

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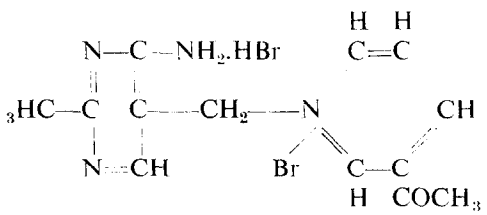
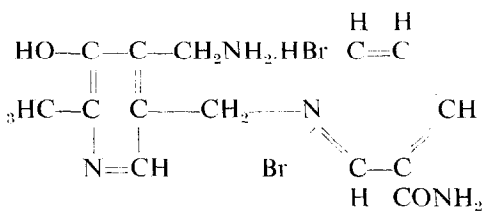


(VII)



(VIII)

Kaplan *et al.*[2] and Makino *et al.*[3] found that mice injected with 3-acetylpyridine (3-AcP) (III) died with paralysis about 8 hr after the injection, whereas the simultaneous injection of niacinamide (NAA) (IV) relieved the animals from the symptoms induced by 3-AcP.

As the thiazol ring resembles the pyridine ring in chemical properties, we first synthesized toxopyrimidyl niacinamide (TXP-NAA) (V), the compound obtainable by substituting the thiazol moiety of vitamin B_1 (VI) with NAA, in order to examine its physiological actions. It was found that NAA prevented the toxicity induced by 3-AcP, the antagonistic action of TXP-NAA competed with 3-AcP in the same way as NAA, and in spite of the presence of the TXP-moiety in the molecule, TXP-NAA caused no convulsion in mice (unlike TXP) even in a dose of 1 mg per g of body

weight. It seemed of interest to test whether TXP-NAA is antagonistic to a compound (TXP-AcP) (VII) obtainable by substituting its NAA-moiety with 3-AcP. Such a compound was synthesized and, unexpectedly, it caused symptoms in mice quite different from those induced by each of the two components (TXP and 3-AcP). A few minutes after the injection of TXP-AcP, the mice had convulsions followed by paresis in the legs, and within 5 min had died. In this case TXP-NAA showed no protective action. NAA, pyridoxamyl niacinamide (PAM-NAA) (VIII) which was obtained by condensing 5-bromopyridoxamine with NAA, and other compounds tested were also found to be of no use, with the exception of phenobarbital. Phenobarbital showed excellent antitoxic action against TXP-AcP. Veronal also had some effect. In order to elucidate the mechanism of action of TXP-AcP we performed the experiments described below.

MATERIALS AND METHODS

In all the experiments DDN male mice weighing from 14 to 15 g were used. TXP was supplied by Takeda Research Laboratories. PAM was supplied by Wakamoto Pharmaceutical Company. TXP-NAA, TXP-AcP and PAM-NAA were synthesized in our laboratory as described below.

Cytochrome *C* (Cyt. *C*) and Cyt. *C* oxidase were prepared from the heart muscle of a cow by the method of Keilin and Hartree,[4] and Eichel *et al.*,[5] respectively. The glutamic decarboxylase was estimated according to Awapara *et al.*,[6] and the Cyt. *C* oxidase according to Eichel *et al.*[5] All the test compounds were dissolved in distilled water and given to mice by intraperitoneal injection.

Synthesis of TXP-NAA

Two hundred milligrams of 2-methyl-4-amino-5-bromomethylpyrimidine dihydrobromide[7] (TXP-Br.2HBr) and 200 mg of NAA were dissolved separately in 2 ml of *n*-butanol and on mixing both the solutions a white precipitate immediately appeared. The mixture was heated to 100–120 °C for 2 hr. At the end of this period, the flask was cooled and placed in a cold room overnight. The resultant amorphous precipitate was filtered, and recrystallized from methanol (m.p. 224 °C to 226 °C; yield 100 mg).

Anal. Calcd. for $C_{12}H_{15}N_5OBr_2.2H_2O$: C, 32·67; H, 4·34; N, 15·87; Br, 36·23; H_2O, 8·16. Found: C, 32·89; H, 4·34; N, 15·28; Br, 36·83; H_2O, 8·07.

Synthesis of TXP-AcP

3-AcP (1·3 g) was dissolved in 2·6 ml of *n*-butanol which contained 1·3 g of 2-methyl-4-amino-5-bromomethylpyrimidine dihydrobromide (TXP-Br.2HBr), and the solution was heated to 110–115 °C for 2·5 hr. About 10 min later, crystals began to separate. At the end of this period, heating was stopped and the flask was left to stand in a cold room overnight. The needle-shaped crystals which separated were filtered, and recrystallized from ethanol (m.p. 206°; yield 2·2 g).

Anal. Calcd. for $C_{13}H_{16}N_4OBr_2.\frac{1}{2}H_2O$: C, 37·80; H, 4·15; Br, 38·69. Found: C, 37·73; H, 4·18; Br, 38·56.

Synthesis of PAM-NAA

Three hundred milligrams of 5-bromopyridoxamine dihydrobromide[8] were dissolved in 3·75 ml of absolute methanol which contained 750 mg of NAA, and the solution was set aside at the room temperature for 96 hr. At the end of this period the precipitate which separated was filtered and washed with 5 ml of absolute methanol.

PAM-NAA thus prepared gave a single spot (*Rf* 0·14) on the paper chromatogram (the solvent system used was the upper layer of a mixture of EtOH, acetic acid, *n*-butanol and water (1:1:4:10, v/v)) under ultra-violet light (Frazar & Hansen Ltd., 301 Clay Street, San Francisco 11, California. U.S.A.). (m.p. 206 °C–207 °C, a hygroscopic compound; yield 78 per cent).

Anal. Calcd. for $C_{14}H_{18}N_4O_2Br_2$: N, 12·91: Br, 36·82; Found: N, 12·56; Br, 35·90.

When 1 mg of PAM-NAA per g of body weight was injected into mice intraperitoneally, the animals did not show any abnormality. Although the administration of 2 mg per g of body weight of the compound did not kill the animals, they nevertheless showed considerable weakness.

RESULTS

The lethal dose of TXP-AcP to mice

As shown in Table 1, the intraperitoneal administration to mice of 550 μg of TXP-AcP per g of body weight usually caused 100 per cent mortality, whereas the lethal doses of 3-AcP and TXP were 0·4 mg and 0·15 mg per g of body weight, respectively. Their molar ratio was 1:1·23:3·05 (TXP:TXP-AcP:3-AcP). The convulsions

TABLE 1. THE LETHAL DOSES OF TXP-ACP TO MICE

Amount of TXP-AcP injected (μg/g of body weight)	Number of animals	Dead/alive
600	5	5/0
550	5	5/0
500	5	3/2
450	5	3/2
400	5	1/4

and running fits in mice caused by the injection of TXP appeared after 1 or 2 hr, and were often repeated. These symptoms and the death of the experimental animals were prevented completely by the injection of B_6-vitamins. In the case of 3-AcP the animals died with paralysis about 8 hr after the injection. The signs of toxicity induced in mice by TXP-AcP were unlike to those caused by TXP or 3-AcP, i.e. some 20 sec after the injection, the animals began to walk about disquietedly. After 1 min they were unable to move their legs smoothly, they waddled and then shivered. They experienced difficulty in breathing, and before long had ceased to move and died quietly after a few minutes. The symptoms were akin to those of the animals administered KCN. If the mixture of TXP and 3-AcP is administered to the mice, the symptoms are a summation of those caused by each component.

Effect of compounds synthesized above on the lethal toxicity of TXP-AcP in mice

1. *Effect of NAA.* As shown in Table 2, NAA did not compete with the TXP-AcP toxicity, even though the intervals of administration of NAA and TXP-AcP, and the dose of NAA were varied.

2. *Effect of TXP-NAA.* As Table 3 shows, neither TXP-NAA nor allithiamine* relieved the animals from the TXP-AcP toxicity. These facts seemed to show that TXP-AcP acted on some site different from that of 3-AcP.

* A disulphide derivative of thiamine with allyl mercaptan.

3. *Effect of PAM-NAA.* The toxicity of TXP was countered by the administration of vitamin B_6, and that of 3-AcP by NAA. PAM-NAA was synthesized as above by the condensation of 5-bromopyridoxamine dihydrobromide (PAM-Br.2HBr) with NAA, and its antagonistic action against the toxicity of TXP-AcP was tested. As shown in Table 4, PAM-NAA showed no clear protective action against the toxicity of TXP-AcP.

TABLE 2. EFFECT OF NAA ON THE LETHAL TOXICITY OF TXP-AcP IN MICE

Amount of NAA injected (μg/g of body weight)	Intervals of administration of NAA and TXP-AcP* (hr)	Number of animals	Dead/alive
50	0·5	3	3/0
50	2	3	3/0
50	4	3	3/0
50	24	2	1/1
130	5	2	2/0
260	5	2	2/0
500	5	2	2/0

* 550 μg of TXP-AcP was usually administered.

TABLE 3. EFFECT OF TXP-NAA AND ALLITHIAMINE ON THE LETHAL TOXICITY OF TXP-AcP IN MICE

Amount of TXP-NAA injected (μg/g of body weight)	Intervals of administration of TXP-NAA and TXP-AcP* (hr)	Number of animals	Dead/alive
332	3	2	2/0
500	3	2	2/0
Allithiamine 500	3	3	3/0

* 550 μg of TXP-AcP was usually administered.

TABLE 4. EFFECT OF PAM-NAA ON THE LETHAL TOXICITY OF TXP-AcP IN MICE

Amount of PAM-NAA injected (μg/g of body weight)	Intervals of administration of PAM-NAA and TXP-AcP* (hr)	Number of animals	Dead/alive
1000	0·5	7	5/2
1000	500 μg, an hour before, and 250 μg, 30 min before the injection of TXP-AcP, and 250 μg simultaneous addition to TXP-AcP	5	3/2
1000	500 μg, 2 hr before, 250 μg, an hour before, and 250 μg, 30 min before the injection of TXP-AcP	5	4/1

* 550 μg of TXP-AcP was usually administered.

Effect of TXP-AcP on mice brain glutamic decarboxylase

About 15 min after the injection of TXP, the mice brain glutamic decarboxylase (GDC) activity decreased to about 60 per cent of normal, and immediately after death it decreased to 40 per cent.[9] In contrast, the brain GDC activity of the TXP-AcP-injected mice was the same as normal: furthermore, in agreement with the *in vivo* experiments, TXP-AcP, even at a concentration of 10^{-2} M, exerted little effect on the normal mice brain GDC activity.

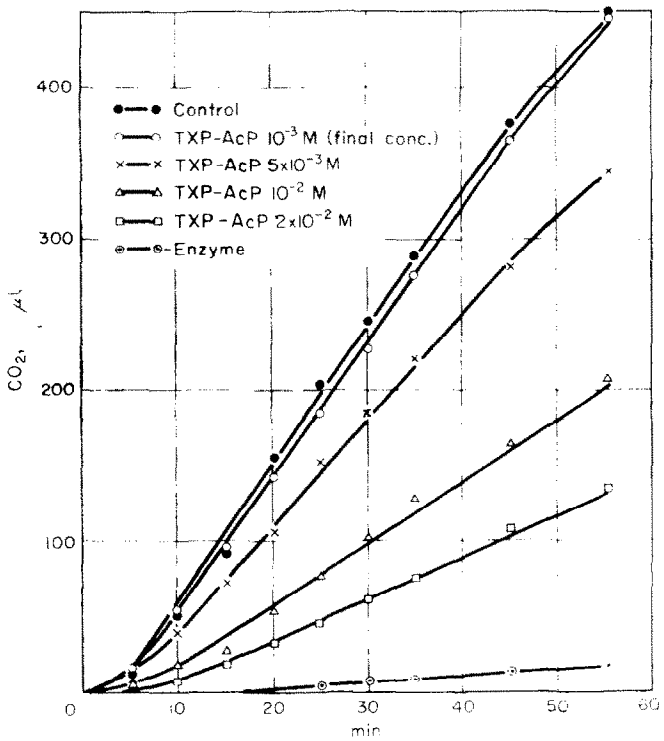


FIG. 1(a). Effect of TXP-AcP on Cyt. C oxidase.

The Cyt. C oxidase activity was estimated according to Eichel *et al.* Experimental conditions: Main compartment of Warburg's vessels, 1·0 ml of Cyt. C oxidase from cow heart muscle, 0·3 ml of 0·1 M semicarbazide (pH 7·1), 0·25 ml of 0·4% Cyt. C solution, varying concentrations of TXP-AcP in 0·5 ml of 0·1 M phosphate buffer (pH 7·1) and 1·1 ml of 0·1 M phosphate buffer (pH 7·1). Side arm, 3 mg of hydroquinone in 0·25 ml of water, 35 °C. After the temperature has reached equilibrium, the content of the side arm was tipped into the main compartment.

Effect on Cytochrome C oxidase

The TXP-AcP-injected mice presented fairly similar symptoms to KCN-injected ones, i.e. both groups of mice died with convulsions in a few minutes. This fact made us think that TXP-AcP might affect the respiratory organs. Though the blood of the mice which died from TXP-AcP poisoning appeared black in comparison with

normal, there was no change in the absorption spectra of the diluted haemoglobin solution. The same is true of KCN-injected mice. It is possible that the TXP-AcP (or KCN)-haemoglobin complex is labile and dissociates in the process of dilution of the blood. Alternatively, the amount of the denatured haemoglobin might be so small as not to be detectable spectrophotometrically. This point is still not clear.

It is believed that KCN inhibits the Cyt. *C* oxidase system. In contrast, the inhibition by TXP-AcP of the Cyt. *C* oxidase system was considerably lower than that of KCN (Fig. 1(a) and 1(b)), i.e. KCN inhibited the enzyme by 52·2 per cent at 10^{-5} M while TXP-AcP inhibited it by 47·5 at 10^{-2} M.

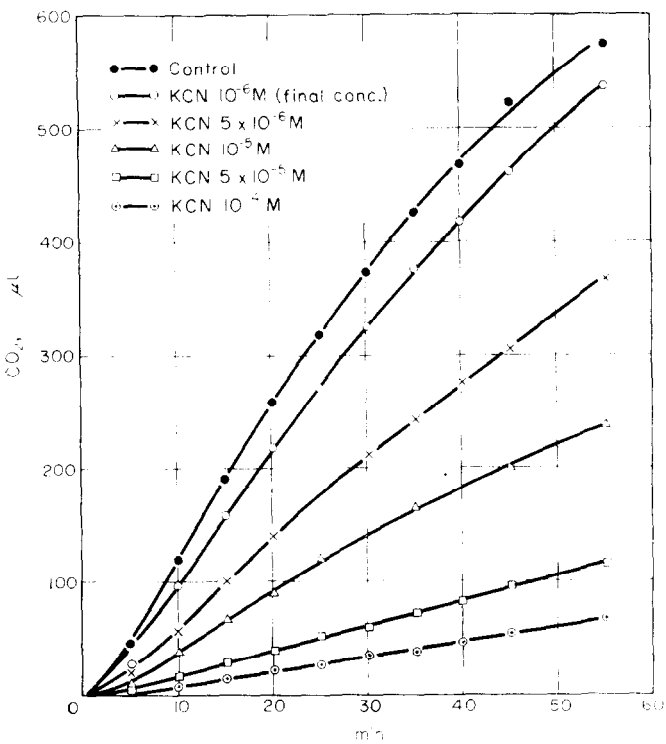


FIG. 1(b). Effect of KCN on Cyt. *C* oxidase.
Experimental conditions are the same as described in Fig. 1(a).

As described later, the symptoms induced by TXP-AcP were countered by the preadministration of phenobarbital while the KCN symptoms were not affected. These data seemed to show that the sites of action of both poisons were different.

Effect of phenobarbital

In all the experiments, 30 min after the injection of phenobarbital TXP-AcP in a dose of 550 μg per g of body weight (lethal dose for mice) was injected into mice intraperitoneally. It was found that from 20 to 55 μg per g of body weight of phenobarbital protected the mice from TXP-AcP toxicity (Table 5). When the amount of

phenobarbital used was over 55 μg, its antagonistic effect to TXP-AcP toxicity was weakened.

Effect of veronal

As shown in Table 6, veronal is also effective against TXP-AcP toxicity, though its effect was a little weaker than that of phenobarbital.

TABLE 5. EFFECT OF PHENOBARBITAL ON THE LETHAL TOXICITY OF TXP-ACP IN MICE

Amount of phenobarbital injected (μg/g of body weight)	Number of animals	Dead/alive
110	5	3/2
55	5	0/5
28	5	0/5
20	5	0/5
14	5	2/3
0	5	5/0

550 μg of TXP-AcP was usually administered 30 min after the injection of phenobarbital.

TABLE 6. EFFECT OF VERONAL ON THE LETHAL TOXICITY OF TXP-ACP IN MICE

Amount of Na-veronal injected (μg/g of body weight)	Number of animals	Dead/alive
50	5	5/0
35	5	3/2
25	8	1/7
17	5	2/3
0	5	5/0

550 μg of TXP-AcP was usually administered 30 min after the injection of veronal.

Effect of artificial respiration

DDN male mice weighing 23 ± 2 g were trained to lie quietly on an animal operating table without anaesthesia. A throat incision was made to expose the trachea for cannulation. A tracheal cannula was inserted routinely and connected to an artificial respiratory apparatus designed for small animals. In all the experiments, the volume and the frequency of the artificial respiration was fixed at 1 ml and at 130–140 times per min, respectively. TXP-AcP was administered by intraperitoneal injection in a dose of 550 μg per g of body weight. The same volume of distilled water was injected

intraperitoneally into the control animals. Five minutes after injection of TXP-AcP, the animals had difficulty in breathing, and artificial respiration was started; at intervals of 5 min it was stopped and the progress of the recovery of respiration was observed. As a result, it was found that difficulty in breathing in mice caused by the injection of TXP-AcP could be countered by artificial respiration, and the mice completely recovered 80 min after the injection. In the control animals nothing unusual was observed during and after artificial respiration.

Moreover, since the intravenous administration of TXP-AcP did not depress the blood pressure of a rabbit anaethetized with urethane, TXP-AcP does not seem to affect the blood circulation system of animals.

DISCUSSION

As indicated above, TXP-AcP which was synthesized in our laboratory, caused violent seizures in mice a few minutes after its intraperitoneal administration. The symptoms differed from those caused by its components, TXP or 3-AcP, and were not countered by the antagonists of TXP or 3-AcP (as described above). In enzymatic experiments, too, the sites of action of TXP-AcP and its components were different from each other. That is, in case of TXP-injected mice, the GDC activity in the brain decreased to about 60 per cent of normal 15 min after the injection of TXP and decreased to 40 per cent after death, whereas, in the case of TXP-AcP-injected mice, it was the same as normal.

According to Kaplan,[2] the toxic action of 3-AcP *in vivo* is due to the 3-AcP analogue of DPN formed by substituting the NAA moiety. Though it had not been confirmed that TXP-AcP was substituted for the NAA moiety of DPN, it seemed for the following reasons that the DPN enzymes are probably not affected by TXP-AcP. First, 3-AcP-injected mice died with paralysis about 8 hr after injection, whereas of the TXP-AcP-injected mice one died in a few minutes with convulsions followed by paresis and dyspnea. Second, the symptoms caused by the injection of TXP-AcP were not countered by NAA, while those by 3-AcP were effectively prevented by the administration of NAA.

The enzymatic experiments showed that the sites affected by TXP-AcP and KCN were different, though the symptoms caused by them were alike.

The TXP-AcP seizures were not prevented by the injection of B_6-vitamins (as in the case of TXP-convulsions) or NAA derivatives (as in the case of 3-AcP), while they were countered by phenobarbital or veronal which are general anti-convulsants. Artificial respiration also prevented death of the mice due to TXP-AcP. From these facts it may be concluded that unlike its components, TXP or 3-AcP, TXP-AcP seems to act directly on the respiratory centre, causing convulsive fits with dyspnea, and further that phenobarbital with its sedative effect inhibits the excitement of the respiratory centre. When the mixture of TXP and 3-AcP was administered to mice, the animals did not show such characteristic symptoms as those caused by TXP-AcP.

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