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SOME ASPECTS OF THE METABOLISM OF TRIAZINE DERIVATIVES ACTIVE IN EXPERIMENTALLY INDUCED VIRUS INFECTIONS

BY

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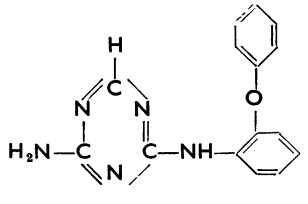
In a recent paper we reported on the metabolism of 4-acetamido-2-morpholino-1,3,5-triazine (Angelucci, Artini, Cresseri, Giraldi, Logemann, Nannini & Valzelli, 1965). In the course of our research we synthesized a great number of symmetrical triazines which were screened in antiviral tests. In doing so, we found another compound, 2-[(*o*-phenoxy-phenyl)-amino]-4-amino-1,3,5-triazine (I), which had an activity even higher than 4-acetamido-2-morpholino-1,3,5-triazine. Compound I is more active in reducing the intensity of pulmonary lesions induced in mice by influenza virus type B. Moreover, this compound, like 4-acetamido-2-morpholino-1,3,5-triazine does not affect the replication of viruses in fertile eggs (Angelucci, 1961 ; Angelucci, Artini, Giraldi, Logemann & Nannini, 1961 ; Angelucci, Artini, Giraldi, Logemann, Nannini & Valzelli, 1963a,b).



I

Consequently, we believe that compound I might have been transformed, *in vivo*, into an active metabolite, and it was interesting to investigate its fate in animals.

METHODS

Radioactive (2-[(*o*-phenoxy-phenyl)-amino]-4-amino-1,3,5-triazine was prepared by labelling the triazine ring with ^{14}C at position 6, according to the method already described (Angelucci *et al.*, 1965). The substance had a specific activity of 100 μc/mM.

In the experiment male and female Wistar albino rats, weighing 200 g on average and fasted for 15 hr, were used. During the experiment the animals were kept in single metabolism cages and were fed the usual laboratory diet after 12 hr from the beginning of treatment. Doses of 28 and 85 mg/kg of the labelled compound, dissolved in 1,2-propandiol, were administered by subcutaneous and intraperitoneal injection to two groups of animals, and a dose of 28 mg/kg,

dissolved in polyethyleneglycol 400, was given intraperitoneally to another group. Measurement of radioactivity in the expired carbon dioxide, and in urine and faeces was made on samples collected at different intervals, 0 to 48 hr after administration; radioactivity in the liver, lungs and kidneys was determined after killing the animals 72 hr after the beginning of the experiment. For the radioactivity dosage the same procedure of sampling and counting described in the earlier paper was followed (Angelucci *et al.*, 1965).

Another group of animals was treated with the labelled compound in order to determine the amount excreted through the bile flow and to detect any possible metabolite. The compound was administered by subcutaneous injection, dissolved in 1,2-propandiol, in a dose of 28 mg/kg. The bile was collected throughout the experiment up to the eighth hour by canalization of the common bile duct in rats anaesthetized with urethane solution injected intraperitoneally. Assessment of the radioactivity dosage was made by the same counting equipment as used previously.

To isolate any possible metabolite 92 ml. of bile removed from another group of animals was treated with 9.2 ml. of 4 N sulphuric acid with cooling to precipitate glycocholic acid. The mixture was left to stand for 2 hr, and then centrifuged. The supernatant fluid was adjusted to *p*H 6.8 with a 32% solution of sodium hydroxide (about 3 ml.) and, after saturation with ammonium sulphate, the supernatant fluid was extracted six times with 40 ml. *n*-butanol. The butanol phases were evaporated to dryness in a vacuum. The residue, an amorphous yellowish mass, weighed 2.5 g, corresponding to 46 mg original triazine on the basis of the radioactivity determination. This residue was dissolved in water (30 ml.) and extracted with ethyl acetate (150 ml.) for 20 hr in a liquid–liquid extraction apparatus. From the organic phase a white powder (32.4 mg) was isolated; this powder, after re-crystallization from glacial acetic acid, showed, by thin-layer chromatography, a R_F higher than that of the triazine compound administered. The aqueous phase was freeze dried and extracted again in Soxhlet by acetone-methanol, 50:1, for 24 hr. Two radioactive fractions were isolated by evaporation; one of them was identical with the one isolated from the ethyl acetate extract, the other was a mixture. In order to identify the latter, a series of chromatographic separations was carried out on 0.2 mm and 2 mm thin-layer of silica gel with a fluorescence indicator (Merck). The acetone-acetic acid mixture (4:1) was used as the mobile phase; Wood's light at 254 mμ and the radioactivity distribution on the chromatographic layer were used for detection. For this purpose, the chromatogram was divided into squares of 1 cm^2 each, and the material of every square was sampled and counted following the procedure described in the previous paper (Angelucci *et al.*, 1965). The use of 2 mm thin-layers was necessary because of the low radioactivity and quantity of the substances examined, particularly in the last phases of the chromatographic separations.

RESULTS

The radioactivity levels are expressed as percentage ratio between the total radioactivity excreted or present in the organs and the radioactivity administered, as well as the equivalent amount of triazine compounds at various times after administration, and they are given in Tables 1, 3 and 4. Table 2 gives the values of 4-acetamido-2-morpholino-1,3,5-triazine (II), published in the previous paper (Angelucci *et al.*, 1965) and shown here to allow an easier comparison with the data for the new compound, indicated as compound I. In Tables 1 and 2, it can be observed that compound I is mainly excreted with the faeces, while compound II is largely eliminated in the urine. Table 4 shows that the concentration of compound I is very high in the bile, which explains its high faecal excretion. Therefore, in order to isolate the metabolism product the bile was the best starting material. In this way we were able to isolate, in a good yield, a compound identified by analysis as the 2-[*o*-phenoxy-phenyl)-amino]-4-amino-6-hydroxy-1,3,5-triazine (compound III), m.p. 315 to 316° (Found: C, 61.17; H, 4.45; N, 23.72; O, 10.84. $C_{15}H_{13}N_5O_2$ requires C, 61.01; H, 4.44; N, 23.72; O, 10.84).

TABLE 1

ELIMINATION OF 2-[(*o*-PHENOXY-PHENYL)-AMINO]-4-AMINO-1,3,5-TRIAZINE IN URINE FAECES AND EXPIRED CARBON DIOXIDE AT DIFFERENT PERIODS AFTER ADMINISTRATION

2-[(*o*-phenoxy-phenyl)-amino]-4-amino-1,3,5-triazine was administered to Wistar albino rats in different ways and dosages. Values are expressed as a percentage ratio between the radioactivity excreted and the radioactivity administered and as mg of compound eliminated

Rats (no.)	Dosage (mg/kg)	Route of administration	Solvent		Excreted in periods (hr) 0–24 Urine	0–48 Urine	0–24 Faeces	0–48 Faeces	0–24 Expired	0–48 CO_2
3	28	Subcutaneous	1,2-propandiol	% of radioactivity administered	9·54	14·59	46·17	61·37	0·30	0·57
				mg of compound eliminated	2·67	4·09	12·93	17·18	0·08	0·16
3	84	Intraperitoneal	1,2-propandiol	% of radioactivity administered	3·97	6·92	38·60	79·32	0·33	0·50
				mg of compound eliminated	3·33	5·81	32·42	66·63	0·28	0·42
3	28	Intraperitoneal	Polyethylene glycol 400	% of radioactivity administered	17·42	21·38	47·04	60·98	0·72	1·48
				mg of compound eliminated	4·88	5·99	13·17	17·07	0·20	0·41

TABLE 2

ELIMINATION OF 4-ACETAMIDO-2-MORPHOLINO-1,3,5-TRIAZINE IN URINE, FAECES AND EXPIRED CARBON DIOXIDE AT DIFFERENT PERIODS AFTER ADMINISTRATION

4-Acetamido-2-morpholino-1,3,5-triazine was administered to Swiss albino mice by stomach tube, suspended in 10% aqueous solution of gum-arabic. Values are expressed as percentage ratio between the radioactivity excreted and the radioactivity administered and as mg of compound eliminated. The animals weighed on average 15 g.

Mice (no.)	Dosage (mg/kg)	Route of administration		Excreted in periods (hr) 0–24 Urine	0–48 Urine	0–24 Faeces	0–48 Faeces	0–24 Expired CO_2
15	328	Oral	% of radioactivity administered	56·6	63·5	19·4	26·7	0·8
			mg of compound eliminated	185·6	208·2	63·6	87·6	2·6

TABLE 3

DISTRIBUTION IN LIVER, LUNGS AND KIDNEYS OF 2[(*o*-PHENOXY-PHENYL)-AMINO]-4-AMINO-1,3,5-TRIAZINE 72 HOURS AFTER ADMINISTRATION

2[(*o*-Phenoxy-phenyl)-amino]-4-amino-1,3,5-triazine was administered to Wistar albino rats. Values are expressed as percentage ratio between the radioactivity present in the organ and the radioactivity administered and as μg/g fresh weight

Groups	Dosage (mg/kg)	Route of administration	Solvent		Liver	Lungs	Kidneys
1	28	Subcutaneous	1,2-propandiol	% of radioactivity administered	0·18	0·05	0·05
				μg/g	1·16	2·76	1·55
2	84	Intraperitoneal	1,2-propandiol	% of radioactivity administered	0·10	0·01	0·02
				μg/g	1·44	1·05	1·56
3	28	Intraperitoneal	Polyethylene glycol 400	% of radioactivity administered	0·06	0·01	0·01
				μg/g	0·45	0·49	0·36

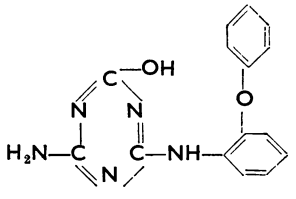
TABLE 4

BILIARY ELIMINATION OF 2(*o*-PHENOXY-PHENYL)-AMINO 4-AMINO-1,3,5-TRIAZINE FROM THE BEGINNING TO 8 HOURS AFTER ADMINISTRATION

2(*o*-Phenoxy-phenyl)-amino 4-amino-1,3,5-triazine was administered to Wistar albino rats. Values are expressed as percentage ratio between the radioactivity excreted and the radioactivity administered and as mg of product eliminated

Rats (no.)	Dosage (mg/kg)	Route of administration	Solvent	% of radioactivity administered	mg of product eliminated
4	28	Subcutaneous	1,2-Propandiol	41·9	11·7

The structure of this compound (III) was verified by synthesis, which was carried out according to a method described by Shapiro, Parrino & Freedman (1959) starting from the corresponding biguanide and ethyl-trichloracetate. The 2-(*o*-phenoxy-phenyl)-amino-4-amino-6-hydroxy-1,3,5-triazine thus obtained had identical mp, mixed mp and



III

infra-red spectrum with the compound isolated from bile. The infra-red spectrum showed ν_{max} 3,470 cm^{-1} (N-H); 3,060, 2,650 cm^{-1} (O-H); 1,600 cm^{-1} (NH_2); and 1,560, 1,450, 792 cm^{-1} (triazine ring vibration).

On the basis of radioactivity measurements made on the separated bile fractions, it is possible to state that in the bile there is 70% of metabolite and 25% of unchanged starting material. Moreover, in chromatograms a radioactive tail between the spots of the two compounds was detected. This tail, after repeated chromatographic separations, proved to be a mixture of the same metabolite and the original triazine, and of another possible compound which, because of its extremely low concentration, could not be identified.

DISCUSSION

The route of administration (intraperitoneal or subcutaneous, is unimportant, because no significant difference exists in the concentration of compound I and its metabolite both in the organs and in the excreted material. At higher doses (84 mg/kg) the faecal excretion increases, while absorption in the organs remains almost unaltered. It is possible to state, therefore, that no cumulation occurs in the organs. It is also interesting to note that compound I, unlike the 4-acetamido-2-morpholino-1,3,5-triazine, is mainly excreted through bile.

While the morpholino ring (compound II) conveys the triazine compound through the kidneys, the diphenyl-ether group (compound I) conveys it through bile. On the other hand, both compounds are metabolized in the same way, both are oxidized at the –CH-group of the triazine ring, introducing an hydroxyl group. This result is in

accordance with the fact that only very little radioactivity was found in the expired carbon dioxide.

Hitherto, the metabolism of symmetrical triazines has not been much described. In plants treated with a herbicide the 2,4-bis-(ethylamino)-6-chlor-1,3,5-triazine (Simazine) the presence of 6-hydroxy-2,4-bis-(ethylamino)-1,3,5-triazine was demonstrated as the first hydrolysis product (Gysin & Knüsli, 1960). Moreover, the triazine ring was not split off. In another study, the metabolism of radioactive triethylenimino-1,3,5-triazine labelled with ^{14}C in the triazine ring was investigated in tumour bearing and control mice. The presence of very little radioactivity in the expired carbon dioxide showed that the triazine ring had been maintained. This was also confirmed by identifying the major urinary metabolite as cyanuric acid. It appears that *in vivo* all the C-atoms of the triazine ring are oxidized and that the triazine ring remains mostly intact (Nadkarni, Goldenthal & Smith, 1954).

SUMMARY

1. The metabolism of 2-[(*o*-phenoxy-phenyl)-amino]-4-amino-1,3,5-triazine has been studied and compared with that of 4-acetamido-2-morpholino-1,3,5-triazine.

2. 2-[*o*-phenoxy-phenyl)-amino]-4-amino-1,3,5-triazine is mainly excreted through bile with the faeces, contrary to 4-acetamido-2-morpholino-1,3,5-triazine which is eliminated largely in the urine.

3. There is no cumulation in the organs studied (liver, lungs, kidneys), because at higher doses the faecal excretion increases while in the same organs the concentration remains unvaried.

4. A metabolite could be isolated from the bile and identified as the 2-[(*o*-phenoxy-phenyl)-amino]-4-amino-6-hydroxy-1,3,5-triazine by comparing it with a sample prepared by synthesis.

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