Brit. J. Pharmacol. (1965), **24**, 274–281.

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

BY

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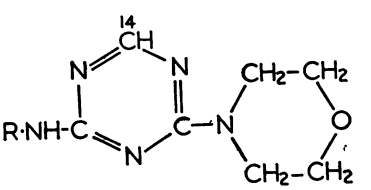
(*Received July* 28, 1964)

An extensive research on the pharmacological action of symmetrical triazine derivatives was carried out in our laboratories and the results obtained in various viral infections, by different experimental tests, have been reported in previous papers (Angelucci, 1961; Angelucci, Artini, Giraldi, Logemann & Nannini, 1961; Angelucci, Artini, Giraldi, Logemann, Nannini & Valzelli, 1963a and b).

Among these compounds the acetylated derivative of 4-amino-2-morpholino-1,3,5-triazine, which can reduce the intensity of pulmonary lesions in mice infected by influenza viruses type B, without affecting, however, the replication of these agents in fertile eggs, appeared to be of particular interest because of its low toxicity (oral LD50 in the rat = 3.48 g/kg; 95% fiducial limits, 2.67 to 4.55). A detailed study of its metabolism was made, therefore, in laboratory animals to ascertain the rate of absorption and the amount of the compound which can be found, after oral administration, in the blood and in various organs (alimentary tract, liver, kidneys) and to investigate the forms in which the compound is present in the organism, that is its metabolites, the possibility of accumulation in the organs, and the rate and the degree of excretion by different routes.

METHODS

Radioactive 4-acetamido-2-morpholino-1,3,5-triazine and 4-amino-2-morpholino-1,3,5-triazine were obtained by labelling the triazine ring with ^{14}C at position 6.



The synthesis of the compounds required the preparation according to Melville, Rachele & Keller (1947) and Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949) of labelled ethyl formate from sodium [^{14}C]formate (Radiochemical Centre, Amersham). By ring closure of anhydro-*N'N'*-bis(2-hydroxyethyl)biguanide with ethyl formate the 4-amino-2-morpholino-1,3,5-triazine (R=H) was obtained, and the

4-acetamido-2-morpholino-1,3,5-triazine ($R=CO.CH_3$) was prepared by *N*-acetylation. The two substances each had a specific activity of 38 μc/mM.

In the experiments on the metabolism of 4-acetamido-2-morpholino-1,3,5-triazine, male Swiss albino mice weighing 15 g were used. For each of the experiments indicated below, three groups of five mice were fasted for 15 hr and kept in individual metabolism cages for the whole experimental period. The animals used for periods longer than 12 hr were fed the usual laboratory diet. Each animal was given, by stomach tube, 22.1 μM of the labelled compound (0.84 μc) suspended in 0.2 ml. of a 10% aqueous solution of gum arabic.

Measurements of radioactivity were made on the carbon dioxide expired in the first 24 hr after the administration of 4-acetamido-2-morpholino-1,3,5-triazine and on samples of blood taken during ether anaesthesia, by section of a carotid artery, at different periods from the beginning of the experiment (from 15 min up to 24 hr). Urinary and faecal radioactivity was measured on samples collected in the periods 0 to 24 and 0 to 48 hr. The radioactivity in the kidneys and in the liver was measured in animals killed at different intervals, from 24 to 240 hr after the administration of the compound, while the radioactivity in the tissues of the stomach and of the small intestine, freed of the materials contained in their lumen by washing with 0.9% saline, was determined in the first hours (from 1 to 6) after the administration.

The measurements of radioactivity in the expired carbon dioxide, urine and faeces were carried out on combined samples from fifteen animals, while the determinations of radioactivity in the blood, kidneys, liver, stomach and small intestine were done on three distinct samples, each corresponding to five animals. The organic materials in the samples were converted to precipitates of barium carbonate by homogenization and wet oxidation according to Rabinowitz (1957). All the samples of barium carbonate were counted at infinite thickness by a thin end-window Geiger counter for a time long enough to ensure counting errors of less than 1% (Geiger counter by 20th Century Electronics, modular scaler SELO, type DCS 415 AU).

Paper chromatographic investigations, intended to show the presence of the original product and of its labelled metabolites, were carried out on the samples of urine collected in the period from 0 to 24 hr after administration of the compound, and on samples of plasma. In order to obtain a sufficiently high radioactivity in the plasma it was necessary to administer a quantity of the labelled product larger than in the experiments already mentioned (110.5 μM, corresponding to 4.2 μc/animal). Blood, withdrawn 1 hr after the administration, from four mice was centrifuged and the plasma was deproteinized according to Cohn, Gurd, Surgenor, Barnes, Brown, Derouaux, Gillespie, Kahnt, Lever, Liu, Mittelman, Mouton, Schmid & Uroma (1950), lyophilized and taken up again with a few drops of warm dimethylformamide. The chromatographic separations were carried out on Whatman No. 1 paper. As reference compound 4-acetamido-2-morpholino-1,3,5-[^{14}C]triazine, 4-amino-2-morpholino-1,3,5-[^{14}C]triazine and [^{14}C]urea were used. The radioactivity on the paper chromatograms was detected with the same counting equipment used for the samples of barium carbonate. The samples of urine were submitted to a preliminary chromatographic separation in a mixture of propanol and water (4 : 1, by volume). The radioactive spots of the chromatograms were extracted in warm absolute ethanol. The concentrated extracts and the samples of deproteinized plasma were then subjected to chromatography with a mixture of butanol, water and acetic acid (4 : 5 : 1, by volume) according to Partridge (1948). The technique did not allow the distinction between the two triazine derivatives; their separation was obtained by extraction of their common radioactive spot on the chromatogram with warm absolute ethanol and by a subsequent chromatographic separation of the extracts with a mixture of butanol, water and concentrated hydrochloric acid (4 : 5 : 1, by volume). In an attempt to clarify some aspects of the metabolism of 4-acetamido-2-morpholino-1,3,5-triazine, the nonacetylated compound, 4-amino-2-morpholino-1,3,5-[^{14}C]triazine, was administered to similar groups of mice, with the same technique described before. These particular investigations were limited to the determination of the radioactivity of samples of blood, taken up to 24 hr after the administration of the compound, and of the urine and faeces eliminated in the first 24 hr, and to paper chromatography of samples of blood.

RESULTS

The radioactivity levels, expressed both as a percentage ratio between the total radioactivity present in the blood (radioactivity/ml. multiplied by the blood volume) and the radioactivity administered, and as the equivalent amount of either of the triazine derivatives,

TABLE 1

BLOOD RADIOACTIVITY LEVELS AT DIFFERENT PERIODS AFTER THE ADMINISTRATION OF 4-ACETAMIDO-2-MORPHOLINO-1,3,5-TRIAZINE

4-Acetamido-2-morpholino-1,3,5-triazine was administered to Swiss albino mice by stomach tube at a dosage of 22·1 μM for each animal. Blood levels are expressed as the percentage ratio between the total radioactivity present in the blood (radioactivity/ml. multiplied by blood volume) and the radioactivity administered, and as μM/ml. of blood. The results are given as means ± standard errors of the values for the three groups each of five animals

	Blood level after									
	15 min	30 min	1 hr	3 hr	6 hr	12 hr	24 hr	48 hr	72 hr	240 hr
% of the radioactivity administered	5·12 ±0·50	5·57 ±0·12	3·82 ±0·19	2·07 ±0·14	0·79 ±0·06	0·54 ±0·07	0·16 ±0·03	0·10 ±0·01	0·05 ±0·01	0·04 ±0·01
μM/ml. of blood	0·769 ±0·075	0·836 ±0·018	0·573 ±0·023	0·311 ±0·017	0·119 ±0·007	0·081 ±0·009	0·024 ±0·004	0·015 ±0·001	0·008 ±0·001	0·006 ±0·001

TABLE 2

BLOOD RADIOACTIVITY LEVELS AT DIFFERENT PERIODS AFTER THE ADMINISTRATION OF 4-AMINO-2-MORPHOLINO-1,3,5-TRIAZINE

4-Amino-2-morpholino-1,3,5-triazine was administered to Swiss albino mice by stomach tube at a dosage of 22·1 μM for each animal. Blood levels are expressed as the percentage ratio between the total radioactivity present in the blood (radioactivity/ml. multiplied by blood volume) and the radioactivity administered, and as μM/ml. of blood. The results are given as means±standard errors of the values for the three groups each of five animals

	Blood level after							
	15 min	30 min	1 hr	3 hr	6 hr	12 hr	24 hr	48 hr
% of the radioactivity administered	4·90 ±0·16	5·09 ±0·14	5·30 ±0·12	2·97 ±0·17	0·95 ±0·17	0·36 ±0·05	0·07 ±0·02	0·03 ±0·001
μM/ml. of blood	0·736 ±0·024	0·764 ±0·021	0·796 ±0·018	0·446 ±0·025	0·143 ±0·025	0·054 ±0·008	0·010 ±0·003	0·005 ±0·001

at various times after their administration, are given respectively in Tables 1 and 2. The blood volume was calculated from the values reported in the literature (Spector, 1956).

It can be concluded from the results in Table 1 that the absorption of 4-acetamido-2-morpholino-1,3,5-triazine is rapid, because after only 15 min the blood levels are very near to the maximum values attained. From Table 2 it appears that the absorption of 4-amino-2-morpholino-1,3,5-triazine is somewhat slower, in that the maximum blood levels are reached after 1 hr instead of after 30 min.

Table 3 shows the values of the radioactivity which were found in the stomach and the small intestine, freed of their contents, at the end of the 1st, 3rd and 6th hr after the administration of 4-acetamido-2-morpholino-1,3,5-triazine. From the diminution of the radioactivity in the walls of the small intestine it may be concluded that absorption is

TABLE 3

DISTRIBUTION IN STOMACH, SMALL INTESTINE, KIDNEYS AND LIVER OF 4-ACETAMIDO-2-MORPHOLINO-1,3,5-TRIAZINE AT DIFFERENT PERIODS AFTER ADMINISTRATION

4-Acetamido-2-morpholino-1,3,5-triazine was administered to Swiss albino mice by stomach tube at a dosage of 22·1 μM for each animal. The content of the various organs is expressed as the percentage ratio between the radioactivity present in the organ and the radioactivity administered, and as μM/g fresh weight. The results are given as means ± standard errors of the values for the three groups each of five animals

		Content after			
		1 hr	3 hr	6 hr	
Stomach	% of the radioactivity administered	1·83 ±0·27	1·41 ±0·19	1·09 ±0·31	
	μM/g	2·722±0·402	2·907±0·283	1·621±0·461	
Small intestine	% of the radioactivity administered	2·75 ±0·12	1·13 ±0·09	0·69 ±0·15	
	μM/g	0·467±0·020	0·192±0·015	0·117±0·025	
		24 hr	48 hr	72 hr	240 hr
Kidneys	% of the radioactivity administered	0·09 ±0·011	0·04 ±0·004	0·03 ±0·003	0·04 ±0·008
	μM/g	0·089±0·011	0·040±0·004	0·030±0·003	0·040±0·008
Liver	% of the radioactivity administered	0·25 ±0·02	0·16 ±0·02	0·09 ±0·02	0·19 ±0·015
	μM/g	0·083±0·007	0·053±0·007	0·030±0·007	0·063±0·005

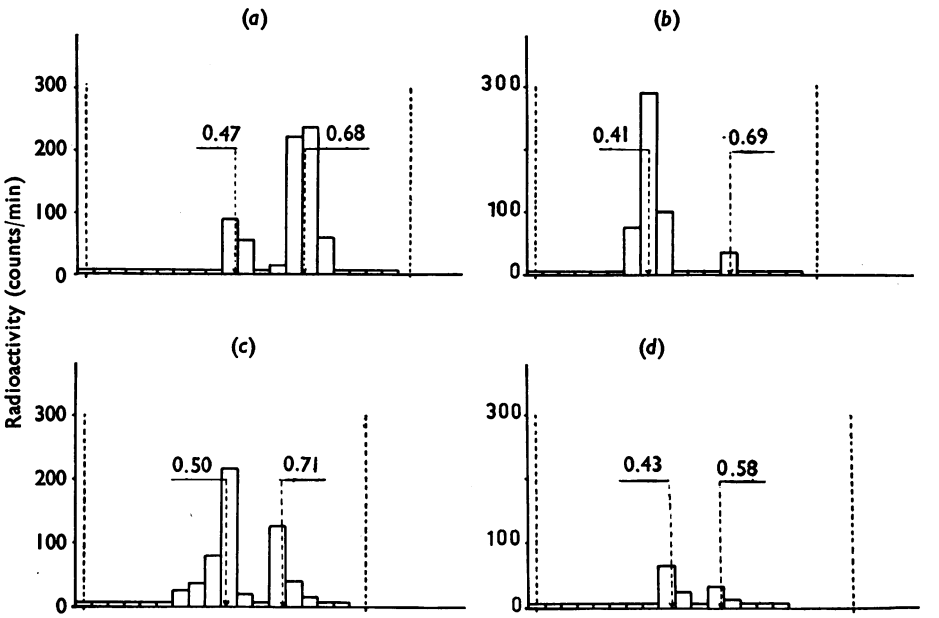


Fig. 1. Distribution of the radioactivity in the chromatograms carried out on samples of plasma and urine of the animals given 4-acetamido-2-morpholino-1,3,5-triazine by stomach tube (110.5 μM per animal). R_F values are given on the histograms. (*a*) Chromatogram of the deproteinized plasma, according to Partridge (1948) (4-amino-2-morpholino-6-hydroxy-1,3,5-triazine R_F=0.47, triazine compounds R_F=0.68); (*b*) chromatograms of the spot with R_F=0.68 of the chromatogram (*a*) rechromatographed with the system butanol : water : hydrochloric acid (4-acetamido-2-morpholino-1,3,5-triazine R_F=0.41, 4-amino-2-morpholino-1,3,5-triazine R_F=0.69); (*c*) chromatogram of the urine according to Partridge (1948) (4-amino-2-morpholino-6-hydroxy-1,3,5-triazine R_F=0.50, triazine compounds R_F=0.71); (*d*) chromatogram of the spot with R_F=0.71 of the chromatogram (*c*) rechromatographed with the system butanol : water : hydrochloric acid (4-acetamido-2-morpholino-1,3,5-triazine R_F=0.43, 4-amino-2-morpholino-1,3,5-triazine R_F=0.58).

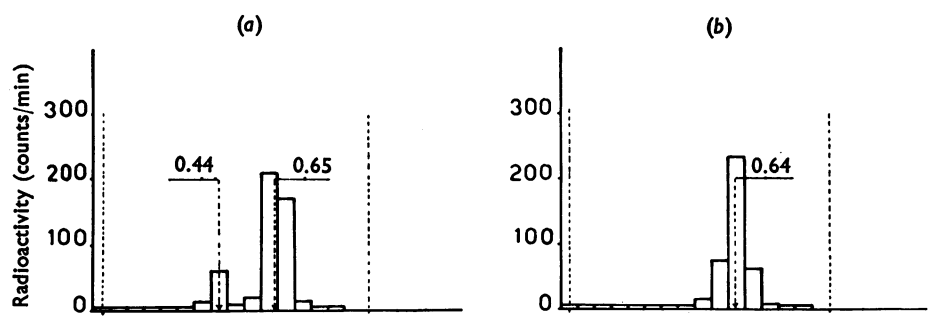


Fig. 2. Distribution of the radioactivity of the chromatograms carried out on the plasma of the group of animals given 4-amino-2-morpholino-1,3,5-triazine by stomach tube (110.5 μM per animal). R_F values are given on the histograms. (*a*) Chromatogram according to Partridge (1948) (4-amino-2-morpholino-6-hydroxy-1,3,5-triazine R_F=0.44, triazine compounds R_F=0.65); (*b*) chromatogram of the spot with R_F=0.65 of the chromatogram (*a*) rechromatographed with the system butanol : water : hydrochloric acid (4-amino-2-morpholino-1,3,5-triazine R_F=0.64).

largely complete within the first 6 hr. In Table 3 are also reported the values for the radioactivity in the kidneys and in the liver at 24, 48, 72 and 240 hr after the administration of the compound. These values are generally small, showing that at least for the two organs considered there is no appreciable accumulation of the product and of its metabolites containing ^{14}C.

The chromatographic investigations of plasma, carried out on samples taken 1 hr after the administration of 4-acetamido-2-morpholino-1,3,5-triazine, showed the presence of the unchanged compound (about 75% of the total radioactivity in the plasma), of the nonacetylated product (about 5%) and of a metabolite (about 20%) with an R_F of 0.47 to 0.50, not significantly different from the R_F of urea (Fig. 1) but which was not, as shown below, urea itself. In the animals treated with 4-amino-2-morpholino-1,3,5-triazine it is possible to show the presence of the identical compound (about 16%) and of the unchanged nonacetylated product (about 84%) (Fig. 2).

The chromatographic investigations on samples of urine excreted in the first 24 hr, from groups of animals treated with 4-acetamido-2-morpholino-1,3,5-triazine, have demonstrated, as in the plasma, the presence of three labelled compounds: the original product (about 23%), the nonacetylated product (about 10%) and the metabolite already demonstrated in the plasma (about two-thirds of the total radioactivity excreted in the urine) (Fig. 1).

In an attempt to identify this last metabolite, the urines were treated with Jack-bean urease; no radioactive carbon dioxide was formed, therefore the metabolite could not be urea. Further and more detailed studies, which we shall report in another publication, have shown that this metabolite is 4-amino-2-morpholino-6-hydroxy-1,3,5-triazine, identical with the synthetic product by melting point, infra-red spectrum and R_F values.

As regards the excretion of the 4-acetamido-2-morpholino-1,3,5-triazine and its metabolites, only a small amount of activity was found in the expired carbon dioxide (0.8% of the amount administered). On the basis of the radioactivity determinations, the main path of excretion appeared to be renal; the urinary values were notably high throughout the first 24 hr (about 57%), while the increase in the succeeding 24 hr was less (about 7%). The fraction eliminated in the faeces was rather low (about 20% in the first 24 hr after the administration of the compound) with an increase (of about 1.8%) in the succeeding 24 hr. From the experiments with 4-amino-2-morpholino-1,3,5-triazine it appears that the urinary excretion in the first 24 hr after administration is clearly lower (about 31% of the total radioactivity) and the faecal elimination relatively higher (about 34%) than in the experiments with the acetylated compound.

DISCUSSION

From the results the following conclusions can be drawn. When administered orally 4-acetamido-2-morpholino-1,3,5-triazine is absorbed with remarkable rapidity and in a notable quantity. This is shown respectively by the blood levels, the maximum of which is reached in a relatively short time after administration, and by high degree of urinary output.

On the basis of the chromatographic investigations, carried out on plasma 1 hr after administration of the compound, it seems certain that 4-acetamido-2-morpholino-1,3,5-triazine is largely absorbed as such. That the absorption in any appreciable degree may be

through a previous deacetylation and a subsequent reacetylation after the absorption can be excluded; in fact, when 4-amino-2-morpholino-1,3,5-triazine was used none of the acetylated product was found in the plasma. However, one cannot exclude the possibility that a fraction of the 4-acetamido-2-morpholino-1,3,5-triazine may not be deacetylated in the alimentary tract and absorbed in this form.

It appears that in the organism an oxidation at position 6 of the triazine ring takes place and 4-amino-2-morpholino-6-hydroxy-1,3,5-triazine is formed.

Another significant result is the very low level of radioactivity found in the kidneys and in the liver 24 hr after the administration of 4-acetamido-2-morpholino-1,3,5-triazine. It may, therefore, be concluded that, at least in these experimental conditions, there is no appreciable accumulation of the original product and of its metabolites labelled with ^{14}C in the two organs examined.

SUMMARY

1. The metabolism of 4-acetamido-2-morpholino-1,3,5-triazine, active in various experimental viral infections, has been studied and compared with that of the corresponding nonacetylated triazine derivative.

2. The compounds, labelled with ^{14}C at position 6 of the triazine ring, were administered to the mouse by stomach tube. The radioactivity levels were determined in blood, expired carbon dioxide, urines and faeces at various times after administration and, for the acetylated compound, also in the stomach, small intestine, kidneys and liver. Paper chromatographic investigations were carried out on 24-hr urines, and on plasma 1 hr after the administration.

3. Blood levels reached a maximum after 15 to 30 min. Radioactivity values in the kidneys and liver up to 240 hr after the administration of the 4-acetamido-2-morpholino-1,3,5-triazine were generally small. In the plasma and urine, chromatography showed a metabolite identified as the 4-amino-2-morpholino-6-hydroxy-1,3,5-triazine. Urinary excretion was high in the first 24 hr.

4. It appears, therefore, that the triazine derivatives are well and rapidly absorbed, and an oxidation at position 6 of the triazine ring takes place. No appreciable accumulation of the products or of their metabolites labelled with ^{14}C has been demonstrated in the kidneys and liver.

The authors are indebted to Dr E. Rivolta, Professor P. L. Cova and Dr M. Romussi of the Sant'Ambrogio Clinic in Milan, for the facilities for part of the radioisotope work.

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