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THE METABOLISM OF ^{32}P-LABELLED TRIETHYLENE-PHOSPHORAMIDE IN RELATION TO ITS ANTI-TUMOUR ACTIVITY

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Ethyleneimines such as triethylenephosphoramide (TEPA, I) and triethylenemelamine (TEM, II) (p. 325) exert remarkable inhibitory effects on the growth of some experimental tumours without seriously impairing the health of the host animal. Total regression of established and actively growing tumours may be achieved (Sugiura and Stock, 1952a and b; Crossley, Allison and Muenzen, 1952; Sugiura and Stock, 1955). TEPA was most effective against the rat tumours R 39 and Jensen sarcoma, less so against the Flexner-Jobling carcinoma and Walker carcinosarcoma 256, and had no action on the Murphy-Sturm lymphosarcoma (Sugiura and Stock, 1955). The Walker carcinosarcoma has been shown to undergo inhibition and then regression, but it later recurs and is then refractory to further treatment (Jackson, 1954). A study of the metabolism of TEPA labelled with radioactive phosphorus was undertaken in the hope of obtaining information about the mode of action of these compounds and of correlating tumour susceptibility with drug fixation in neoplastic tissue.

METHODS

Preparation of Triethylenephosphoramide-^{32}P.—The method used was based on that described by Bestian (1950) for the preparation of the unlabelled compound. Phosphorus oxychloride (3 g.) labelled with ^{32}P (20 mc.) was diluted to 15 ml. with dry benzene and added slowly to a well-stirred mixture of ethyleneimine (2.52 g.) and triethylamine (5.92 g.) in dry benzene (20 ml.). $POCl_3 + 3NH(CH_2)_2 + 3N(C_2H_5)_3 \rightarrow PO[N(CH_2)_2]_3 + 3N(C_2H_5)_3.HCl$. Vigorous stirring was continued for an hour before removal of the precipitated hydrochloride. The filtrate was concentrated to 2–3 ml. *in vacuo* and centrifuged to remove a small amount of solid. Aliquots of the clear supernatant fluid were cautiously distilled *in vacuo* in a micro-distillation apparatus. If the oil is heated above 130° it is liable to decompose violently. The distillate (b.p. 90° C. at 0.3 mm.) was collected on a central tube cooled with solid CO_2. It crystallized as a colourless hygroscopic solid which was stored in sealed containers at −20° C.

The purity of the labelled compound was assessed by ascending paper chromatography on Whatman No. 1 paper. Two solvent systems, water-saturated *n*-butanol and 80% ethanol, were used, and gave single, well-defined peaks with R_F values of 0.57 and 0.8 respectively.

Animal Techniques.—The distribution of radioactivity after a single injection of labelled TEPA was studied in rats of an American Wistar strain (100–150 g.) implanted with the Walker carcinosarcoma 256, or bearing a resistant variety of the same tumour developed by treatment with TEM (Jackson, 1954). The distribution after repeated injections (five consecutive daily doses) was studied in rats of the same strain implanted with the Jensen sarcoma. Freshly prepared aqueous solutions of labelled TEPA (specific activity 2,000 counts/min./μg.) were injected intraperitoneally at a dose of 1.0 mg./kg. No significant toxic effects occurred after five daily injections of this dose. Groups of animals were killed with ether at selected times. Blood was removed by ventricular puncture and diluted with 3.8% sodium citrate solution. Various tissue samples were then removed, care being taken to avoid cross-contamination during dissection. In each experiment a zero time was selected and all estimates of radioactivity were corrected to this time. As a standard procedure in the digestion of solid tissues for liquid counting, each weighed sample was treated with 2–3 ml. of lithium hydroxide solution (10% lithium hydroxide in 20% ethanol) and diluted to 10 ml. with water. Estimations of radioactivity were made in a liquid counter tube with an efficiency of about 7% for ^{32}P.

In determining the percentage excretion of the administered activity urine was collected from a group of five animals. Small amounts of urine were collected on a sheet of plastic cloth placed immediately beneath the cage. By feeding the animals with milk the degree of contamination of urine with faeces was greatly diminished. Aliquots of the urine (about 25 μl.) were chromatographed directly.

RESULTS

Anti-tumour Activity.—The inhibitory effect of TEPA on the growth of the Walker carcinosarcoma (5-day-old tumours) and its resistant form is shown in Table I. Six daily doses of labelled TEPA (2 mg./kg., i.p.) commencing 24 hr. after implantation of the Walker tumour resulted in complete inhibition for nearly three weeks. The tumour then grew in most animals. This effect is similar to that previously described for TEM (II) on the same tumour (Jackson, 1954). However, 6-day-old Jensen rat sarcomata, similarly treated, regressed completely (Table I) and there was no recurrence even after nine months.

Tissue Distribution After a Single Injection of Labelled TEPA.—Two groups of rats, implanted with the normal Walker tumour and its resistant variety respectively (tumour weights about 10 g.), were given the radioactive drug (1.0 mg./kg.). Samples of blood, plasma, spleen, and tumour all showed a rapid clearance of the drug in the first 4 hr. (Table II).

No significant difference was detected in the distribution of radioactivity in animals bearing the two varieties of Walker tumour. The mean tissue levels in a group of five normal rats at 24 hr. were 0.16%, 0.03%, and 0.21% of the administered activity/g. of blood, plasma, and spleen respectively. These values were similar to those of the tumour-bearing rats at the same time. Most of the administered radioactivity (80–90%) was present in urine collected during the first 24 hr. after injection.

Tissue Distribution after Repeated Injection of Labelled TEPA.—Rats bearing the Jensen sarcoma (tumours about 10 g.) were given five daily doses of labelled TEPA (1.0 mg./kg., i.p.). The tissue radioactivity was measured 24 hr. after the final dose (Table III), and again no appreciable retention of radioactivity was found. At the same time, the very low plasma radioactivity compared with that of the red cells and tissues suggested

TABLE I

THE INHIBITORY EFFECT OF TEPA (6 DAILY DOSES, 2 MG./KG., I.P.) ON THE GROWTH OF THE WALKER CARCINOSARCOMA 256, A RESISTANT FORM OF THE SAME TUMOUR, AND THE JENSEN SARCOMA

In all groups ten animals were used. The times given refer to the number of days after implantation of the tumours.

	Walker Tumour		Resistant Walker Tumour		Jensen Sarcoma	
	Controls	Treated	Controls	Treated	Controls	Treated
Day treatment began	—	4	—	4	—	6
Day experiment ended	11	11	11	11	21	21
Tumour wt. (g.)	28	1·8	39	22	22	0
	24	1·2	31	18	14	0
	22	1·0	23	13	12	0
	22	0·9	23	13	12	0
	21	0·9	21	12	11	0
	20	0·7	20	11	9·7	0
	18	0·5	20	7·5	9·3	0
	17	0·5	19	2·8	3·3	0
	14	0·4	17	2·8	0	0
	10	0·1	6·0	0·5	0	0

TABLE III

THE ^{32}P CONTENT OF VARIOUS TISSUES FROM RATS BEARING THE JENSEN SARCOMA (TUMOUR WT. ABOUT 10 G.) 24 HR. AFTER FIVE DAILY INJECTIONS OF LABELLED TEPA (1.0 MG./KG., I.P.)

Each value is the mean of five samples, the range being indicated in parentheses

	Counts/min./g.	% of Total Dose/g. Tissue
Blood	293 (270–322)	0·020
Plasma	18 (11–27)	0·001
Kidney	461 (432–494)	0·031
Liver	642 (605–700)	0·043
Spleen	546 (530–614)	0·036
Muscle	215 (177–240)	0·014
Testis	176 (134–198)	0·012
Tumour	330 (305–347)	0·022
Fat	108 (44–186)	0·007

TABLE II

TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER ONE INJECTION OF LABELLED TEPA (1.0 MG./KG., I.P.)

Two groups of rats were implanted with the Walker carcinosarcoma and its resistant variety respectively. Each value is the mean of 5 samples, the range being indicated in parentheses. Results are expressed as the percentage of administered dose present/g. of tissue

	Hr. after Administration				
Walker tumour	0·5	1·5	4	24	48
Blood	0·78 (0·72–0·81)	0·50 (0·47–0·53)	0·18 (0·16–0·19)	0·11 (0 09–0·12)	0·08 (0·06–0·11)
Plasma	0·77 (0·74–0·79)	0·49 (0·36–0·64)	0·19 (0·14–0·25)	0·01 (0·005–0·017)	0·01 (0·008–0·013)
Spleen	0·69 (0·63–0·76)	0·49 (0·48–0·50)	0·23 (0·19–0·28)	0·20 (0·18–0·23)	0·12 (0·11–0·18)
Tumour	0·72 (0·68–0·75)	0·52 (0·49–0·57)	0·24 (0·17–0·27)	0·15 (0·13–0·22)	0·08 (0·07–0·11)
Resistant Walker tumour	0·5	1·5	3·5	12	24
Blood	0·69 (0·51–0·82)	0·45 (0·36–0·50)	0·18 (0·14–0·21)	0·08 (0·06–0·10)	0·11 (0·09–0·13)
Plasma	1·16 (0·65–1·50)	0·71 (0·54–0·76)	0·15 (0·10–0·18)	0·01 (0·008–0·013)	0·01 (0·006–0·013)
Spleen	0·66 (0·48–0·78)	0·49 (0·41–0·59)	0 23 (0·18–0·26)	0·21 (0·19–0·26)	0·21 (0·17–0·26)
Tumour	0·63 (0·44–0·76)	0·49 (0·41–0·54)	0 21 (0·14–0·23)	0·12 (0·07–0·16)	0·14 (0·10–0·16)

that some reaction of the drug with cellular components had occurred. Tumours fractionated chemically by the method of Davidson and Smellie (1952) showed no specific localization in particular cellular components. Thus, in the Jensen tumours referred to above the distribution of radioactivity was as follows: trichloroacetic acid soluble material, 32%; lipoids, 23%; nucleic acids, 25%; proteins, 20%. Crystallization of the haemoglobin from the blood samples and separation of the pigment into haematin and globin according to the method of Anson and Mirsky (1930) showed that over 90% of the ^{32}P was associated with the protein fragment. No such fixation of radioactive phosphorus occurred after the administration of repeated doses of labelled phosphate.

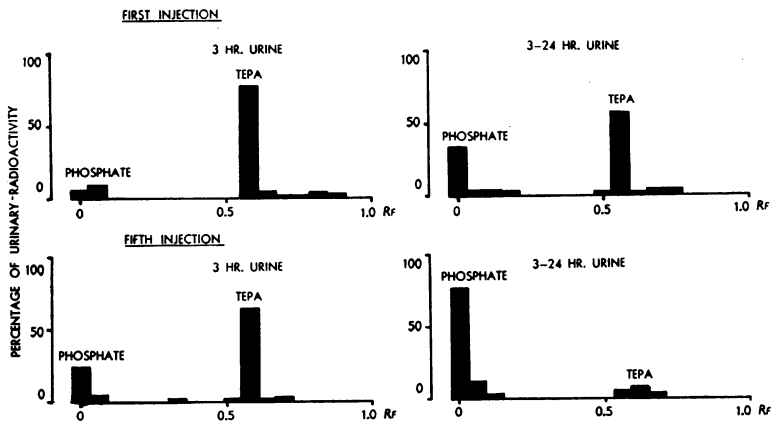


FIG. 1.—Chromatograms showing the proportions of phosphate and TEPA present in urine from rats bearing the Jensen sarcoma. Direct chromatography of samples collected 3 hr. and 24 hr. respectively after the first injection (upper graphs), and fifth injection (lower graphs), of radioactive TEPA (1.0 mg./kg., i.p.). The solvent system was water-saturated butanol. The increase in the proportion of inorganic phosphate in the 3–24 hr. urine is due to its slow excretion compared with unchanged TEPA. This is most apparent in urine collected 3–24 hr. after the 5th injection.

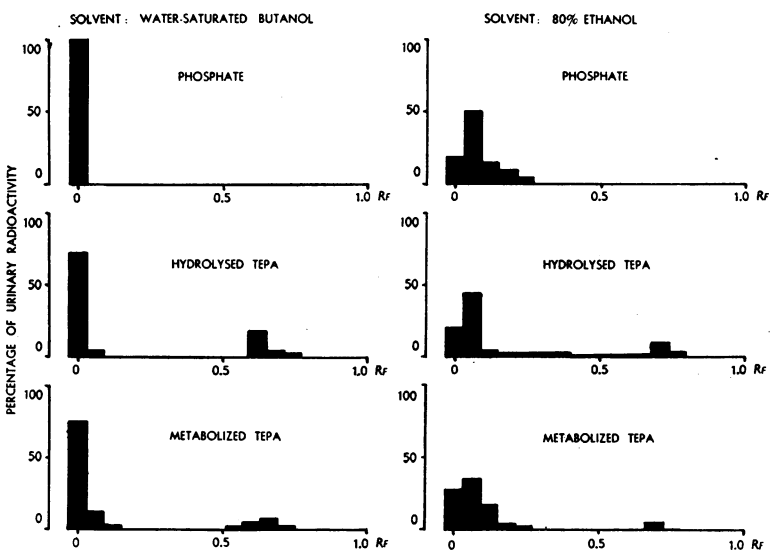


FIG. 2.—Chromatograms showing the similarity between labelled phosphate and the *in vivo* and *in vitro* breakdown products from radioactive TEPA. Hydrolysed TEPA was prepared by heating an aqueous solution (1 mg./ml.) at 95° for 24 hr. The metabolic components were present in rat urine collected 24 hr. after the last of a series of five doses of labelled TEPA (1.0 mg./kg., i.p.)

Metabolism of TEPA in Rats.—The chromatographic distribution of ^{32}P in the urine of animals given labelled TEPA was determined. Preliminary investigation of 24 hr. urine samples from treated animals bearing Walker tumours had shown that urine could be satisfactorily chromatographed with the solvent mixtures used for assessing the purity of labelled TEPA. These experiments had also indicated that a substantial portion of the drug was excreted unchanged. This was investigated more fully in animals with Jensen sarcomata during a course of 5 daily injections of the labelled drug. Urine was collected 3 and 24 hr. after each injection. Samples of the urine were chromatographed immediately. In 3 hr. samples 50–70% of the radioactivity was from unchanged drug, the rest of the radioactivity being located near the starting line (Fig. 1). Chromatograms of the 3–24 hr. urine samples showed a relative increase in the amount of metabolized phosphoramide which, in the last specimen, comprised 90% of the activity (Fig. 1).

The deterioration of an aqueous solution of the labelled TEPA was followed chromatographically. The product closely resembled the metabolite

found in urine. In view of the similar location of phosphate on chromatograms, the hydrolysis product from TEPA, and its principal urinary metabolite, appears to be inorganic phosphate (Fig. 2). Colorimetric estimations on aqueous solutions of TEPA also showed a slow liberation of inorganic phosphate.

Discussion

Cytotoxic agents like ethyleneimines and nitrogen mustards are believed to exert their pharmacological action by chemical interaction (alkylation) with unknown cell components vital to cell division. One is impressed by the emphasis laid on the chemical reactivity of these compounds. Thus Skipper, Bennett, and Langham (1951) using ^{14}C-labelled methyl-*bis*-(2-chloroethyl)amine found that after 24 hr. 80% of the administered radioactivity remained widely distributed and fixed throughout the tissues. Nevertheless, brief reference has been made to the absence of any specific localization of TEM (II) labelled with ^{14}C in the triazine ring (Skipper, 1953). Recently a more detailed account of the metabolism of this labelled compound in normal and tumour-bearing mice has appeared (Nadkarni, Goldenthal, and Smith, 1954). Following a single large dose (2 mg./kg.) about 80% was excreted in 24 hr., and chromatographic examination of the urine showed that no unchanged drug was present. The tissue radioactivity was widely distributed, and again no specific localization was observed. Chromatographic analysis of the 24 hr. urine revealed only one radioactive metabolite, which was identified as cyanuric acid (III). The presumption is that the ethyleneimino-groups were removed by a metabolic process and it was suggested that the biological activity of TEM might be related to a small portion of intact drug retained within the body. The rapid elimination of this drug is in agreement with previous experiments which suggested that its effect is short-lived, like that of the nitrogen mustards. Thus Goldberg and Schoenbach (1951) found that simultaneous injection of TEM and inoculation of mice with tumour inhibited the growth of the latter, whereas injection of the drug one hour before inoculation failed to hinder development of the tumour.

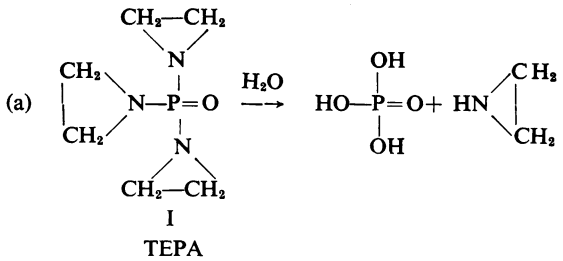
TEPA has been previously labelled with radioactive phosphorus and its distribution in rats examined (Crossley, Allison, Wannemacher, Migliarese, Parker, Kuh, Seeger, and Partridge, 1953). It was administered in a single large dose (about 25 mg./kg., s.c.) to normal rats and to rats bearing the Flexner-Jobling carcinoma, a susceptible tumour. Various tissues were examined up to 30 hr. after the dose, but no noteworthy selective concentration in normal or tumour-bearing rats was observed. About 65% of the radioactive dose was accounted for in the urine in 24 hr. In the plasma, 2 hr. after injection, the majority of the radioactivity (60%) was found by these authors to be precipitated with the globulin fraction, but no other data on the distribution in blood were given. No criteria of the purity of the TEPA used were published.

In earlier experiments of the present series, using TEPA known to be partially polymerized, a large fraction of the plasma radioactivity was also precipitated with the proteins. This did not occur in subsequent experiments when pure, labelled TEPA was used, although the tissue distribution pattern was in substantial agreement with the data of Crossley *et al.* (1953). The radioactive compound used in these later experiments was also shown to be pharmacologically active after the tracer studies had been completed, when it produced inhibition and temporary regression of the Walker tumour. These points are stressed since the small-scale preparation of pure radioactive TEPA is difficult because of its tendency to polymerize when distilled. Paper chromatography was found to be a good method of establishing its purity.

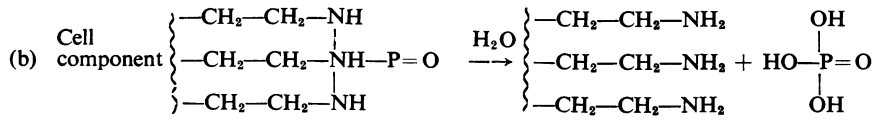
The precipitous fall in plasma radioactivity (Table II) and the recovery of over 80% of the dose in the 24 hr. urine show that TEPA is rapidly excreted. In all the tissues examined the amount of ^{32}P was greatest in the first sample (½ hr.), after which it fell rapidly at rates comparable to that in plasma. This correlates with the high proportion of unchanged radioactive TEPA present in urine, especially in the early stages (Fig. 1). This latter observation is in sharp contrast with the behaviour of labelled TEM which is completely metabolized in the mouse (Nadkarni *et al.*, 1954).

The idea that ethyleneimines are generally highly reactive alkylating agents under physiological conditions is not favoured by our results, which also suggest that only a small proportion of the drug produces the anti-tumour effect. It seems likely that the influence of the drug is exerted on a highly susceptible process in a sensitive tumour, for only a minute part of the dose is retained and there is no selective localization of radioactive material. Chromatographically, the only metabolite found in urine appears to be inorganic phosphate (Fig. 2), which might be produced by

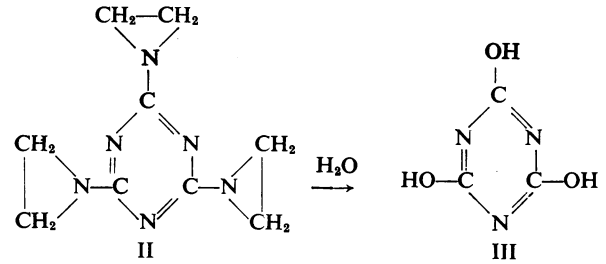
simple hydrolysis (a) or subsequent to reaction of the compound with tissue components (b):



I
TEPA



The *in vivo* production of inorganic phosphate from TEPA parallels the formation of cyanuric acid from TEM described by Nadkarni *et al.* (1954):



II
TEM

III
Cyanuric acid

It is curious, however, that the majority of the TEPA escapes from the rat unchanged whereas TEM in mice at a comparable dose (2.0 mg./kg.) was found to be completely metabolized to cyanuric acid.

The separation of various tumour components after injection of labelled TEPA has not indicated localization of radioactive material in any particular cell fraction. Since the compound is partly metabolized into phosphate, the question arises whether the radioactivity fixed in the tissues represents combined drug. It is possible that localization of TEPA by combination with cell components is followed by breakdown of the complexes formed with the liberation of phosphate as illustrated above. The radioactive phosphate could then enter locally into the many cellular reactions involving phosphate, and account for the distribution found. The observed localization of radioactivity in the protein part of haemoglobin supports the view that the reaction of TEPA with cellular material is not necessarily followed by the loss of phosphate. More certain information of the site of reaction of the ethyleneimine group could be obtained by the use of a labelled atom in this three-membered ring.

Summary

1. Triethylenephosphoramide (TEPA) labelled with radioactive phosphorus has been prepared, its purity established chromatographically, and its anti-tumour activity confirmed.

2. Only a small part of the administered radioactivity was retained by the animal after 24 hr. No specific localization of radioactive material occurred in any rat tissue examined after doses which effectively inhibited the Walker carcinosarcoma and Jensen sarcoma. In tumour tissue the radioactivity was not associated with any particular cell component.

3. Over 80% of the injected dose was excreted in the urine in 24 hr. The majority of this was present as unchanged drug. The only metabolite detected was identified chromatographically as inorganic phosphate.

4. Although TEPA is a potent tumour inhibitor it does not appear to be very reactive chemically *in vivo*. The results suggest that its cytotoxic activity may be due to the interference by a very small proportion of the dose administered with some highly susceptible cellular mechanism.

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