Biochemical Pharmacology, 1959, Vol. 3, pp. 42–50. Pergamon Press Ltd., Printed in Great Britain.

METABOLIC STUDIES OF ^{32}P-LABELLED TRIETHYLENETHIOPHOSPHORAMIDE

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(*Received* 23 *May* 1959)

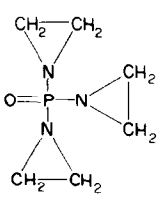
Abstract—Triethylenethiophosphoramide (ThioTEPA) labelled with radioactive phosphorus has been prepared and its metabolism examined in the rat, mouse, dog and rabbit. The drug was metabolized rapidly *in vivo*. The primary stage was the rapid replacement of S by O with the formation of TEPA. The mouse was exceptional in that the compound was degraded so that the principal radioactive metabolite excreted was inorganic phosphate In the rat, TEPA was the main metabolite; but the dog and rabbit excreted three other metabolites, although the major product was TEPA.

There was no specific localization of labelled material in the tissues of the mouse and rat apart from the retention of some radioactivity in the protein part of rat haemoglobin. The results suggest that this type of alkylating agent, in spite of its biological activity is not highly reactive chemically *in vivo*.

INTRODUCTION

IN AN earlier study of the distribution of ^{32}P-labelled triethylenephosphoramide (I) in the rat no specific localization of the drug was found in any of the tissues.[1] Over 80 per cent of the radioactive material appeared in the urine in 24 hr mainly as unchanged drug, suggesting that TEPA, a potent tumour inhibitor, was not very reactive chemically *in vivo*. These results contrasted with later findings by other workers which showed that labelled TEPA was virtually completely metabolized in the mouse to inorganic phosphate with no unchanged drug excreted.[2] In a recent paper on the distribution and fate of alkylating agents,[3] it was concluded that the excretion of a large proportion of unchanged TEPA in the rat "is incongruous in view of the postulated high degree of chemical and biological reactivity of this group of alkylating drugs".

In order to obtain more information on the fate of this type of compound we have examined the metabolism of ^{32}P-labelled triethylenethiophosphoramide (II) in the rat, mouse, dog and rabbit. Some preliminary results have been published elsewhere.[4]



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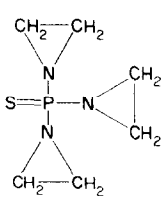


II

MATERIALS AND METHODS

Preparation of P-*labelled triethylenethiophosphoramide*

Phosphorus trichloride, labelled with ^{32}P (35 mc), was converted to phosphorus sulphochloride.[5] To 2 g of the phosphorus trichloride, 0·04 g of aluminium trichloride and 0·465 g of sulphur were added. On heating, an exothermic reaction occurred and the product was distilled from the resultant clear brownish solution. The phosphorus sulphochloride was a colourless liquid, b.p. 122 °C (1·8 g). This product, dissolved in benzene (20 ml) was slowly added to a cooled mixture of triethylamine (3·22 g) and ethyleneimine (1·5 g) in dry benzene (20 ml), with stirring and cooling to 5 °C. After 30 min the mixture was filtered to remove precipitated triethylamine hydrochloride, the filtrate concentrated *in vacuo* and the solid residue recrystallized from benzene–petroleum ether (b.p. 40–60°). It formed colourless crystals (1·6 g), m.p. 50 °C and was chromatographically homogeneous in butanol: dioxan: 2 N ammonia ($R_f = 0·9$). Its specific activity was $1·76 \times 10^4$ counts/min per μg.

Distribution studies

The tissue distribution of radioactivity after a single injection of labelled thioTEPA (2·0 mg/kg, i.v.) was made in adult male rats of an American Wistar strain and in male mice. Freshly prepared aqueous solutions of the labelled compound were injected either intravenously or intraperitoneally. At selected times, groups of animals were exsanguinated by cardiac puncture under ether, the blood being collected in heparinized tubes and stored at 4 °C. Various tissue samples were removed and weighed. As a standard procedure solid tissues were digested for liquid counting, each weighed sample being heated with 2–3 ml of a solution of lithium hydroxide (10 per cent in 20 per cent aqueous ethanol) before diluting to 10 ml with water. The liquid counter tube had an efficiency of 9·1 percent for ^{32}P.

Metabolic studies

Dogs were anaesthetized with pentobarbitone (50 mg/kg), their ureters catheterized and urine samples collected as required after intravenous injection of the drug. Metabolic cages were used to collect urine from rabbits, rats and mice. In rats and mice killed soon after injection, however, urine was taken directly from the bladder.

Chromatography

Plasma and urine samples were chromatographed without pre-treatment. Single dimension ascending paper chromatography on Whatman no. 1 paper was used throughout. The main solvent system employed was freshly prepared butanol: dioxan: 2 N ammonia (4:1:5 v/v). The distribution of radioactivity on chromatograms was accurately determined with a multi-counter system[6] as well as by contact autoradiography using Ilford X-ray film.

RESULTS

Tissue distribution

Details of the distribution of radioactive material in the tissues of the rat and mouse are given in Table 1. In the mouse, a higher percentage of the dose was present in kidney tissue than in the rat—other mouse tissues contained less radioactivity, which corresponded with the more rapid clearance of the drug in this animal. The blood

level fell very rapidly, for 2 min after injection only 14 per cent of the administered radioactivity remained in the circulation of the rat and 10 per cent in the mouse. Similar low levels (less than 10 per cent of the radioactive dose) were found in the dog and rabbit a few minutes after injection. Blood from rats killed 9 days after injection contained a significant amount of radioactivity associated with the red cells. Examination of the haemoglobin from these cells, followed by separation of the haem from the globin, showed that all the ^{32}P was attached to the globin part of the molecule. A similar effect had been observed previously in rats given repeated injections of labelled TEPA.[1]

TABLE 1. TISSUE DISTRIBUTION OF RADIOACTIVITY IN THE RAT AND MOUSE AFTER INTRAVENOUS INJECTION OF LABELLED THIOTEPA (2 mg/kg). THE 30 MIN, 200 MIN AND 9 DAY VALUES IN BOTH SPECIES ARE THE MEANS FROM FIVE ANIMALS

Time after injection	Percentage of dose present at: 2 min	5 min	10 min	15 min	30 min	200 min	9 days
Rat							
Blood	14·2	11·7	10·7	9·8	8·3	4·5	0·53
Plasma	7·9	5·7	5·7	5·7	4·7	2·5	0·002
Spleen	0·36	0·51	0·47	0·62	0·44	0·26	0·09
Kidney	0·75	0·73	0·66	0·76	0·53	0·39	0·07
Liver	11·3	8·0	5·7	6·0	4·4	3·2	0·27
Testes	0·86	0·90	0·91	1·06	0·78	0·69	0·09
Mouse							
Blood	10·0	—	9·9	10·2	8·7	1·7	0·05
Plasma	4·7	5·6	5·5	5·6	5·1	1·0	0
Spleen	0·27	0·50	0·51	0·51	0·34	0·14	0·01
Kidney	2·0	2·8	2·6	2·7	3·1	1·2	0·06
Liver	6·8	8·9	7·8	8·7	7·8	3·9	0·20
Testes	0·41	0·44	0·62	0·60	0·43	0·17	0·02

Metabolism of thioTEPA

(1) *Rat.* Chromatography of urine collected $\frac{1}{2}$, 2 and 24 hr after injection of thioTEPA showed that the radioactivity was almost entirely present as a single spot ($R_f = 0{\cdot}7$), corresponding exactly to labelled TEPA in the same solvent system. In the 24 hr sample two other labelled radioactive materials were present ($R_f = 0{\cdot}05$ and $R_f = 0{\cdot}15$) each representing about 5 per cent of the total radioactivity (Fig. 1). Chromatography of plasma 30 min after injection showed approximately equal proportions of unchanged drug and metabolite, $R_f = 0{\cdot}7$; 200 min after administration no unchanged material was present, the majority of radioactivity (75 per cent) being located in the TEPA zone ($R_f = 0{\cdot}7$) and the remainder about the origin (Fig. 1).

(2) *Mouse.* Urine samples collected $\frac{1}{2}$ and 3 hr after injection of the drug were examined. The majority of radioactivity was present near the origin of chromatograms (Fig. 2), a position occupied by inorganic phosphate in the same solvent system. The remainder was spread out on both chromatograms from R_f 0·1 to 0·5 with a small peak at $R_f = 0{\cdot}4$–$0{\cdot}5$. Chromatography of plasma taken 30 and 200 min after injection revealed only a trace of unchanged drug; two components at $R_f = 0{\cdot}7$ (TEPA) and $R_f = 0{\cdot}05$ (phosphate) accounted for most of the radioactivity present (Fig. 2).

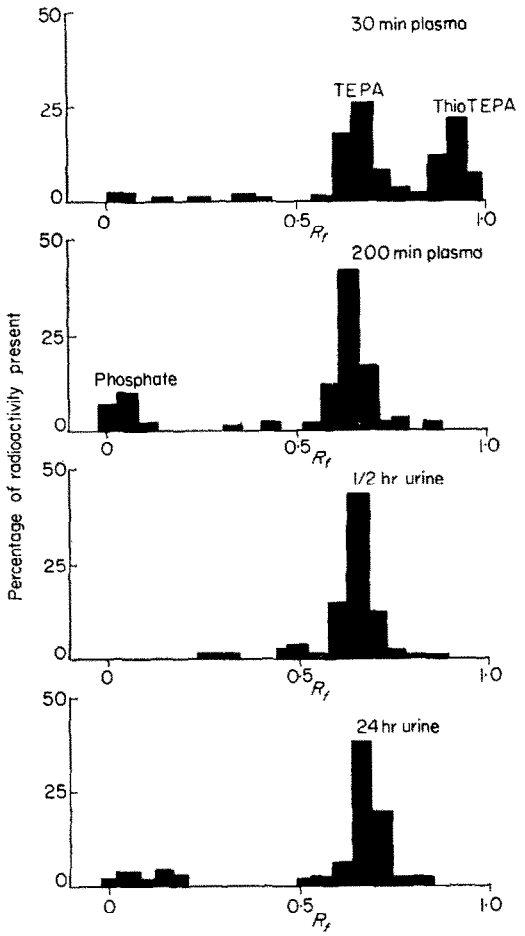


FIG. 1. Chromatograms showing the proportions of radioactive components present in plasma and urine from rats injected with radioactive thioTEPA (2 mg/kg, i.v.).

(3) *Dog*. A more detailed investigation was made of the radioactive materials present in plasma and urine shortly after injection of the labelled drug. Only urine samples collected less than 30 min after injection contained any unchanged drug, the main constituent being chromatographically identical with TEPA; three other components, were also present (R_f = 0·05, 0·2 and 0·5,) (Fig. 3). Because the blood radioactivity fell so rapidly chromatography of plasma had to be restricted to samples collected during the first $\frac{1}{2}$ hr after injection. The main component was TEPA although the metabolite, R_f = 0·5, found in urine, was also detected.

(4) *Rabbit*. Urine was collected for several days after injection, when 70 percent of the radioactive dose was accounted for in the combined samples (Table 2). The radioactive metabolites present were similar to those found in the dog (Fig. 3). In later samples the proportion of phosphate present increased but the major metabolite remained as TEPA (Table 2).

TABLE 2. THE PROPORTION OF RADIOACTIVE METABOLITES PRESENT IN RABBIT URINE AT DIFFERENT TIMES AFTER THE INJECTION OF THIOTEPA (2 mg/kg, i.v.). THE AMOUNTS ARE EXPRESSED AS PERCENTAGES OF THE RADIOACTIVE DOSE, THE METABOLITES BEING SEPARATED BY CHROMATOGRAPHY IN BUTANOL-DIOXAN 2 N AMMONIA (4:1:5 v/v).

Time (hr)	Radioactivity present (%age dose)	Composition of urine (as %age dose)			
		R_f 0·05	R_f 0·2	R_f 0·5	R_f 0·7
0–3	3·2	0·1	0·4	1·1	1·3
3–5	15·7	1·7	2·3	3·6	6·7
5–21	27·3	5·5	4·1	5·2	10·7
21–29	20·3	6·1	2·6	3·3	7·7
29–45	1·6				
45–74	1·2				
0–74	69·3	13·4	9·4	13·2	26·4

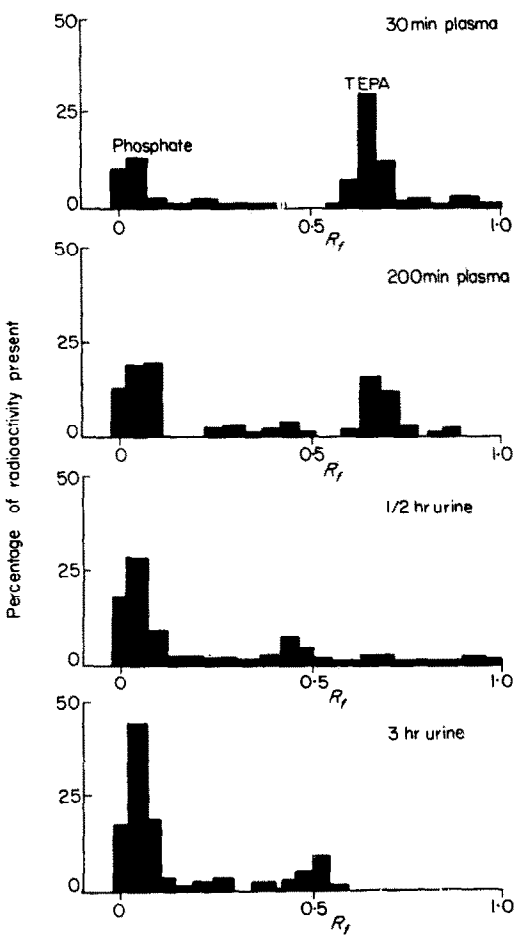


FIG. 2. Chromatograms showing the proportions of radioactive components present in plasma and urine from mice injected with radioactive thioTEPA (2 mg/kg, i.v.).

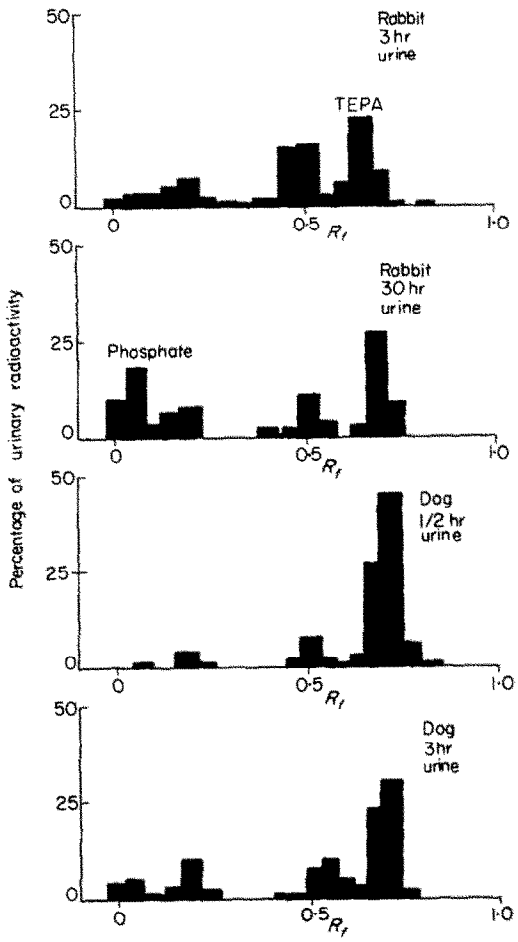


FIG. 3. Chromatograms showing the proportions of radioactive metabolites in the urine of dogs and rabbits injected with radioactive thioTEPA (2 mg/kg, i.v.).

Stability of thioTEPA. Samples of mouse and rat urine containing added thioTEPA, 8, 80 and 800 μg/ml of urine, were incubated at 37 °C. Chromatographic examination showed no deterioration of the drug after incubation for 2 hr; even after 20 hr only about 5 per cent had been hydrolysed to phosphate.

Excretion of ^{32}P-phosphate

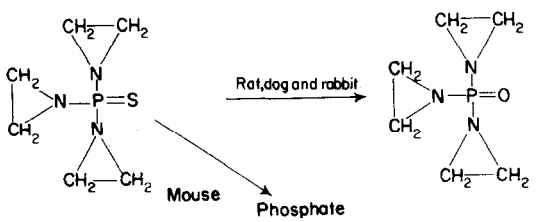
Five mice and five rats were injected with a solution of ^{32}P-labelled phosphate (1 mg/kg). Urine collected for 18 hr after injection contained about 14 per cent of the radioactive dose in each species.

DISCUSSION

The mechanism by which compounds containing ethyleneimine groups produce their biological effects is probably chemical reaction with cell components (alkylation). Other types of radiomimetic agents are known to react *in vivo* in this

way. For example, the aliphatic nitrogen mustard, methyl bis(2-chloroethyl)amine, labelled with ^{14}C, reacted rapidly in mice, 30–50 per cent being fixed in 6 hr.[7] The rate of excretion was correspondingly slow, the 24 hr urine containing less than 10 per cent of the radioactive dose. It may be presumed that most of the drug was bound in locations which are biologically unimportant. Attempts to study the reaction of alkylating agents with proteins and nucleic acids have been made *in vitro* but experimental conditions are very different from those *in vivo*.[8]

In our experiments using labelled thioTEPA, the rapid drop in circulating radioactivity after intravenous injection and the speed with which the tissues reached comparable specific activities indicate the ease with which the drug was distributed throughout the body. In the rat, 70 per cent of the radioactive dose was excreted in 18 hr, almost entirely as TEPA (Fig. 1). In the dog and rabbit the metabolic picture was more complicated, since four metabolites were separated; the major component, however, was TEPA (Fig. 3). A radical degradation of the drug occurred in the mouse with inorganic phosphate as the main metabolite although the plasma picture indicated that the first stage is the production of TEPA. In the urine there was no unchanged drug and only a trace of TEPA even $\frac{1}{2}$ hr after injection; later urine samples were similar in composition (Fig. 2). Compared with the other species examined the mouse is remarkable in its ability to metabolize the ethyleneimino-compound completely (Fig. 4). The phosphorus label provides no clue to the fate of the nitrogen rings, although earlier work in the mouse with ^{14}C ethyleneimino-labelled TEM suggests a general breakdown rather than a detoxication by alkylation of specific substances.[9] It is clear that the biologically active alkylating groups may remain largely unreacted in some species and so be excreted unchanged. In fact, the greater tolerance of the mouse for such compounds may be connected with ability to metabolize them rapidly.



In the mouse about 90 per cent of the radioactive dose of thioTEPA was excreted in 18 hr. Since only 14 per cent of a comparable dose of radioactive phosphate was recovered in the same period of time, a mechanism is indicated by means of which phosphate formed from the drug does not enter the general phosphate pool in this species. The inference is that the degradation of TEPA occurs in the mouse kidney—a point which is being further examined. Mouse urine, incidentally, does not possess the capacity to liberate phosphate from thioTEPA *in vitro*.

The only evidence of selective retention of labelled material was found in the red cells of rats (Table 1); its location was in the globin part of haemoglobin. This fixation of radioactivity in the protein part of haemoglobin had previously been noted after the injection of ^{32}P-labelled TEPA.[1] The drug appears to be directly involved in this

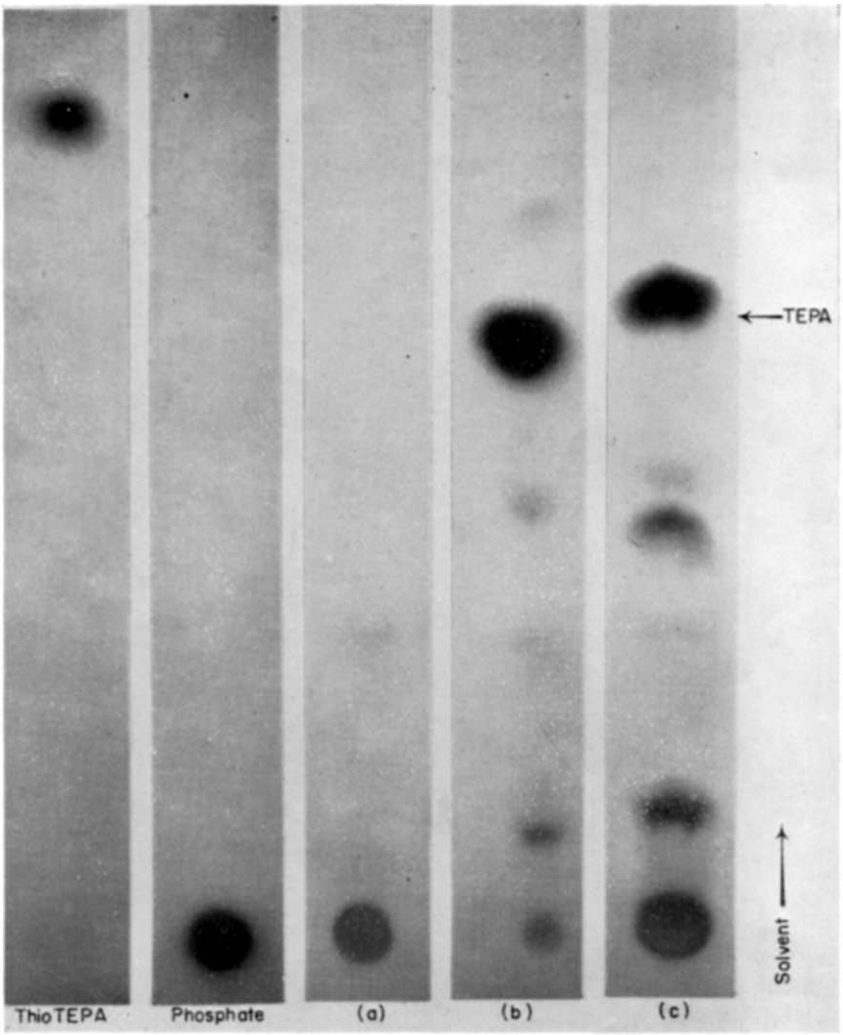


FIG. 4. Autoradiographs of chromatograms showing species differences in the radioactive metabolites present in urine collected 2 hr after giving ^{32}P-labelled thioTEPA (2 mg/kg, i.v.). Autoradiographs *A*, *B* and *C* are of mouse, rat and dog urine, respectively. For comparison the behaviour of ^{32}P-labelled thioTEPA and of phosphate are shown, using the same solvent system (butanol:dioxan:2 N ammonia).

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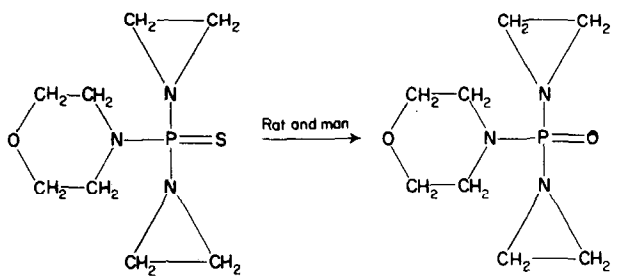
reaction with protein, for an equivalent dose of labelled phosphate did not result in the formation of labelled haemoglobin.

Species differences in the metabolism of thioTEPA reconcile the conflicting reports on the metabolism of TEPA. Our results with thioTEPA in mice agree with the known conversion of this substance to inorganic phosphate in this species.[2] A general assessment of the *in vivo* reactivity of ethyleneiminophosphoramides based on their fate in the mouse is therefore misleading. The metabolism of ethyleneimine compounds, such as thioTEPA, is more typically represented in other species where the excreted metabolites contain unreacted ethyleneimine groups. The metabolism in man and the rat of morpholinodiethylenethiophosphoramide (III), labelled with ^{32}P and ^{14}C, has been studied.[10] After injection of the drug into rats a considerable proportion of radioactive material in the urine (66 per cent of the 24 hr sample) was identified as morpholinodiethylenephosphoramide (IV), i.e. the sulphur had been replaced by oxygen. Using ^{14}C-morpholinodiethylenethiophosphoramide (III) the chief metabolite in man also was the desulphurized form (IV); only a trace of morpholine was present, and other ^{14}C compounds present were not identified.[10]



III IV

Besides their antitumour activity, ethyleneimine compounds are known to produce marked effects on haematopoiesis[11] and spermatogenesis.[12] The evidence suggests that, although these compounds are widely distributed throughout the body, they are not highly reactive chemically and the marked biological effects are the result of the action of minute amounts of drug at specific locations. This type of compound, unlike more reactive kinds of alkylating agent, may ultimately yield information on the cell components involved in their biological effects provided a sufficiently detailed study of *in vivo* reaction sites can be made.

Acknowledgements—This work has been supported by grants made to this hospital by the British Empire Cancer Campaign.

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