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SYNTHETIC AMOEBICIDES: PART III. ESTIMATION OF 6-AMINOANTHRAPYRIMIDINE IN TISSUES: METABOLISM AND EXCRETION

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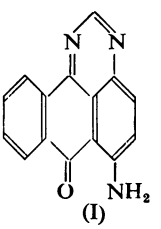
In a previous paper in this series (Jones, Landquist, and Senior, 1952) it was shown that 6-aminoanthrapyrimidine (I.C.(P) Code No. 3074) and the related compounds such as the 6-dialkylaminoalkylamines had a distinct curative action in experimental amoebiasis in young rats when tested by the method of Jones (1946). Of these, 3074, a readily obtainable substance (I), proved to be



(I)

more active than Chiniofon (7-iodo-8-hydroxyquinoline-5-sulphonic acid). It was highly active *in vitro* against *Entamoeba histolytica*, though less active than emetine in both methods of evaluation. It was well tolerated in rats, and the chemotherapeutic index was of the order of 30 to 33. Jones (personal communication), however, found that 3074 had a pronounced photodynamic action when albino rats and mice dosed with it were exposed to sunlight. In order to throw light on this effect a fluorimetric method of estimating 3074 in tissues has been devised, and a study has been made of its distribution, metabolism, and excretion in rabbits. A few tests have also been performed in rats.

EXPERIMENTAL

Estimation of 6-Aminoanthrapyrimidine (3074)

The substance 3074 occurs as orange yellow needles, m.p. 272–4° C. (uncorr.), when crystallized to constant melting point from xylene. It is very sparingly soluble in water (50 μg. per 100 ml. at 18° C., 250 μg. per 100 ml. at 37° C.), but rather more soluble in organic solvents, the solution in non-polar solvents exhibiting a strong green fluorescence. The greenish-yellow colour is insufficient for direct colorimetric estimation except in high concentrations. It is not practicable to diazotize the amino-group and then to form an azo compound capable of colorimetric estimation. Moreover, no colour reaction is given with either *p*-dimethylaminobenzaldehyde or β-naphthaquinonesulphonic acid.

The green fluorescence in benzene is, however, sufficiently intense to serve as a basis for analysis in the range of concentrations found in experimental animals. Extraction of tissue homogenates is very nearly complete, provided that the medium is saturated with sodium chloride; the blank values from fresh tissues are of a low order. When blood concentrations are below 10 μg./100 ml. the method is less satisfactory owing to blank interference, but this does not affect the findings recorded here, since relatively high concentrations were met with in all tissues.

Reagents

Standard Solution of 3074: 25 mg. dissolved in 40 ml. glacial acetic acid and diluted to 100 ml. with distilled water. This solution, which is stable, is diluted 1 in 250 with water to form the standard solution (1 ml. contains 1 μg.).

Benzene: Commercial benzene, the fluorescence of which has been removed by distillation through an efficient fractionating column; the first and last portions (each about 10%) are rejected.

0.2M-*Disodium Hydrogen Phosphate Solution:* Reagent purity.

Sodium Chloride: Reagent purity.

Apparatus

Spekker photo-electric absorptiometer used as a fluorimeter. Filter Green W.7. Both photocells, of 10 ml. capacity, were used.

Procedure

(1) *Standard Curve.*—A standard solution which gives a concentration of 5 μg. 3074 in 10 ml. of the benzene extract serves as a satisfactory standard against which unknown dilutions may be compared. Quantities of 10, 8, 6, 4, 3, 2, and 1 ml. of the standard solution are pipetted into 60-ml. glass-stoppered bottles and adjusted to 10 ml. with distilled water; an eighth bottle

contains water only; 5 ml. sodium phosphate solution is added to each bottle followed by excess sodium chloride; 20 ml. benzene is added by burette and the bottles shaken vigorously for 10 minutes. The benzene layer is decanted into tubes of at least 25 ml. capacity and centrifuged (1,500–2,000 r.p.m. for 10 minutes). Using these quantities, the top standard contains 5 μg. of drug in 10 ml. of benzene extract.

(2) *Estimation of Tissue Concentration.*—Blood or plasma (5 ml.) or homogenized tissue (5 ml. containing 0.5 g. of tissue) is pipetted into 10 ml. of water contained in a 60-ml. glass-stoppered bottle; sodium phosphate solution (5 ml.) is then added, followed by excess sodium chloride, and the procedure as for the standard curve is followed. It is important to use centrifuge tubes of at least 25 ml. capacity in order to obtain 10 ml. of benzene solution free from solid matter. After centrifugation, any emulsion which forms in the benzene layer is stirred with a glass rod and the tube re-centrifuged; a clear benzene layer then results with all tissues. If it is found necessary to dilute the benzene extract, distilled benzene, previously shaken with saturated brine and filtered, should be used.

(3) *Estimation of Unchanged Drug in Urine.*—Urine, or a suitable dilution (1 ml.), is pipetted into a 60-ml. glass-stoppered bottle containing sodium phosphate (5 ml.) and water (10 ml.). Benzene (20 ml.) is added and the bottle is shaken for ten minutes. The benzene layer is centrifuged before the reading is taken. Dilution of the benzene layer with centrifuged water-saturated benzene may be necessary.

(4) *Estimation of Unchanged Drug in Faeces.*—The daily output of faeces is comminuted by pestle and mortar, ground with water, and made up to 1,000 ml. A suitable portion (1 to 10 ml.) is then treated as in (3) above.

Notes.—(1) Drying the solvent after extraction or the addition of acetic acid (1%) to the extract enhances the fluorescence, but these refinements have not been adopted, for the improvements are of little practical value.

(2) Sodium phosphate is used in order to retain the fluorescent constituents of the tissues in the aqueous phase. Blank tests on blood carried out in the absence of this reagent illustrate its success in this respect. A high blank fluorescence is occasionally found in faeces, but the drug concentration is such that the accuracy of the method is little affected.

(3) The necessity for salting out with sodium chloride arises from the low recoveries otherwise obtained from blood and other tissues; urine, however, gives a satisfactory mean value of 99%. Ten recoveries from whole blood (horse) give an average of 72.5% without salt and an average of 103% with salt. Corresponding figures for rabbit are 71% and 102%.

The mean recoveries ± the standard deviation, obtained by adding various amounts of the standard solution of 3074 to homogenized rat tissues or excreta and following the analytical procedure described, were: blood, 102±3.0%; serum, 94±3.4%; liver, 82±2.6%; kidney, 83±1.2%; spleen, 94±2.0%; lung, 78±1.4%; muscle, 86±3.4%; fat, 89±1.7%; faeces, 94±2.7%; urine, 99±3.3%. About 10 analyses (9–11) were performed in each group.

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Experiments on Rabbits

Determination of Blood Concentration.—Young chinchilla rabbits of either sex, weighing from 2.0 to 2.7 kg., were used. A single 100-mg./kg. dose was given orally as a dispersion in Dispersol O.G. (I.C.I.) and a sample of blood (about 5 ml.) was taken from the ears at suitable intervals (Table I). The animals were kept in subdued light to prevent skin sensitization. Urine and faeces were collected separately, at the same time; the urine was collected in flasks containing 1% mercuric chloride solution (1 ml.) and protected from light in order to minimize any possible photochemical decomposition. The urine was stored at 5° C. until required for analysis.

TABLE I

BLOOD CONCENTRATIONS AT VARIOUS INTERVALS FOLLOWING A SINGLE 100-MG./KG. ORAL DOSE IN RABBITS

Hours after Administration	Blood Concentration (μg. per 100 ml.) for Individual Animals				Mean Value
	1	2	3	4	
1·16	213	170	241	586	302
3·5	582	390	254	612	460
6·0	950	324	206	204	421
24	800	337	790	162	522
48	30	38	96	88	63
72	20	13	0	0	16
96	0	0	0	0	0

Tissue Distribution.—A rabbit receiving one 100-mg./kg. oral dose was killed after 16 hours, and an estimate was made of the total amount of free drug present in the tissues and in the lumen of the alimentary tract (Table II).

Search for Urinary Metabolites.—A preliminary examination was made of the first day's urine from a rabbit receiving the 100-mg./kg. dose. During the analytical procedure for unchanged drug it was observed that the diluted urine, after benzene extraction, was more highly pigmented than normal urine, being of a deep yellow-brown colour. A water-soluble metabolite was suspected, and qualitative tests on the diluted residual urine confirmed this view. Heating the urine with dilute hydrochloric acid followed by neutralization with excess sodium bicarbonate and subsequent extraction with benzene gave a solution with a green fluorescence similar in appearance to 3074. At this stage it was thought that the water-soluble metabolite was probably a conjugated form of 3074 which reverted to the parent substance on acid hydrolysis. The

TABLE II

DISTRIBUTION OF DRUG IN RABBIT 16 HOURS AFTER ONE 100-MG./KG. ORAL DOSE. RABBIT WEIGHT 1·7 KG.

Tissue	Weight (grammes)	Concentration (mg. per 100 g.)	Total (mg.)	Percentage of Administered Dose
Blood[1]	110	0·49	0·54	0·32
Liver	62	2·2	1·36	0·71
Kidney	14	0·63	0·088	0·05
Spleen	0·6	1·1	0·007	0·004
Lung	8·3	1·07	0·089	0·052
Muscle[1] ..	68	0·56	0·380	0·220
Fat[1]	10	0·22	0·022	0·013
Brain	8	0·95	0·076	0·045
Eyes	4·3	0·57	0·023	0·013
Stomach ..	—	—	24·60	14·50
Duodenum ..	—	—	0·67	0·40
Caecum and ileum	—	—	0·72	0·42
Large intestine ..	—	—	0·15	0·09
Faeces	None	—		
Urine[2]	120	(Free) 0·75	0·90	0·53
		(Conjugated[3]) 1·98	2·38	1·4
Total ..			32·0	18·8

[1] The weights of these are estimated. [2] Including bladder contents. [3] This is described below.

following test was carried out to ascertain whether 3074 was quantitatively liberated from the water-soluble metabolite. Urine (1-ml. portions of a 1/4 dilution), which had previously been freed from unchanged drug by four extractions with equal volumes of benzene, was heated in a boiling-water bath for one hour with various concentrations of hydrochloric acid (1 ml.). Excess sodium bicarbonate was added to neutralize the acid and the usual procedure of benzene extraction and estimation was followed. Concentrations of acid ranging from 1.4N to 7.5N obviously gave complete liberation of free drug, since values between 1.8 and 2.0 mg. per 100 ml. were obtained. A concentration of 0.3N-hydrochloric acid gave only 0.6 mg. per 100 ml., and a blank estimation without acid gave 0.1 mg. per 100 ml. A further test revealed that quantitative liberation of the green fluorescent substance in the cold was caused by 6.6N-hydrochloric acid within two minutes. The following experiments were performed in order to identify this liberated metabolite.

Fourteen rabbits were each given three doses of 100 mg./kg. of 3074 on alternate days, and the urine, collected daily in the presence of mercuric chloride (approx. 1 in 20,000) was pooled, treated with $Na_2HPO_4.12H_2O$ (50 g. per litre), and stored at 0–5° C. until required. The urine collected over a period of six days measured 12.7 litres and was of a reddish-brown colour. A portion of the urine (1,000 ml.) was extracted with half its volume of distilled benzene and the yellow fluorescent extract was evaporated to 200 ml. and purified chromatographically on alumina (Spence grade 0) which had previously been freed from water-soluble alkali by extraction with boiling 5% acetic acid followed by washing and reactivation.

The experiment was conducted in subdued light and the column examined in the ultra-violet. The bulk of the dissolved substance was strongly adsorbed, but a narrow band which exhibited a yellow fluorescence was fully eluted with benzene. This fraction upon evaporation gave a trace of gummy material and was rejected. On introducing methanol (0.5% v/v gradually increased to 10%) the brown substance developed into a yellow fluorescent band, leaving a dark-brownish and non-resolvable ring at the top of the column. The yellow fluorescent eluate was evaporated to dryness, giving 20 mg. of a yellowish brown non-crystalline deposit, m.p. 260–4°. Crystallization from methanol gave 15 mg. of brown-coloured needles, m.p. 270–1°. Further crystallization gave a substance, m.p. 272–4°, which gave no depression of melting point on admixture with a pure sample of 6-amino-anthrapyrimidine, m.p. 272–4° (the melting points are not corrected). Analysis had shown that 25 mg. of unchanged drug was present in the volume of urine taken, the recovery of 15 mg. thus amounting to 60% of the theoretical.

A 10-litre quantity of the urine was freed from benzene-soluble drug by repeated benzene extraction and the concentration of hydrochloric acid was brought to normality by the addition of the concentrated acid. The mixture was then heated on a steam bath for one hour. The cooled solution was treated with excess sodium bicarbonate and extracted with benzene (two portions of 3 litres). This extract, which was washed with brine, was of a rich reddish-brown colour with a strong green fluorescence. Removal of the solvent left a dark-brown semi-crystalline material weighing 2.63 g. which could not be purified by crystallization; it was redissolved in benzene (700 ml.) and purified chromatographically on alumina. Elution with 0.5–2% methanol gave a brown diffused band bordered with a yellow fluorescence and a greyish-brown material remained at the top of the column. A narrow blue-purple fluorescent band followed the major band, but this was not investigated. The brown band was cut from the column and dried. Soxhlet extraction with methanol followed by fractional crystallization gave both needles (m.p. 269–271°; yield 0.53 g.) and prisms (m.p. 269–272°; yield 0.2 g.) which gave no depression of melting point on admixture. Further crystallization of the combined yield gave 0.55 g. of brown needles, m.p. 274°. There was no depression of melting

point on admixture with pure 6-aminoanthrapyrimidine, m.p. 272–4°. *Analysis:* Found C, 72.5; H, 3.85; N, 17.15. $C_{15}H_9ON_3$ requires C, 72.8; H, 3.6; N, 17.0%.

Analysis of the water-soluble metabolite in the volume of urine employed (*vide infra*) gave the equivalent of 1.03 g. of water-soluble drug calculated as 3074, and the recovery of 0.73 g. corresponds to 71% of this amount.

Estimation of the Water-soluble Metabolite.—The observation that the water-soluble metabolite was converted to 3074 on treatment with hydrochloric acid provided a basis for quantitative analysis, and the following technique was adopted.

Method.—The urine, or a suitable dilution (1 ml.), was pipetted into a boiling tube containing N-hydrochloric acid (10 ml.) and was mixed by means of a glass rod and immersed in a boiling-water bath for one hour. The cooled solution was transferred to a 60-ml. glass-stoppered bottle with the aid of a minimum amount of water (5 ml.) and slowly made alkaline with sodium bicarbonate of reagent purity (2 g.). Benzene (20 ml.) was added, and the procedure now followed the method for unchanged drug. The value for total drug was thus determined, and the value for water-soluble drug, calculated as the equivalent of 3074, was obtained by subtraction.

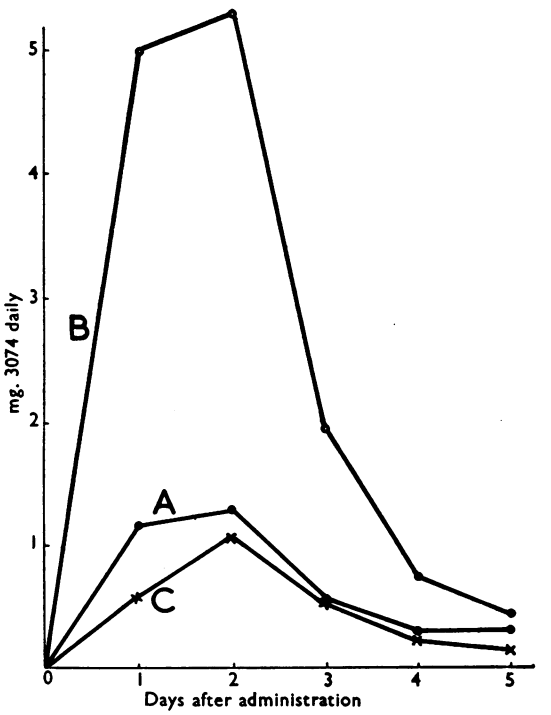

FIG. 1.—Daily excretion in four rabbits following 100-mg./kg. single oral doses of 3074, calculated as mg. per day from one 2-kg. rabbit. A is urine, unchanged drug; B urine, water-soluble drug; C is faeces, unchanged drug.

Excretion.—After a single 100-mg./kg. oral dose in four rabbits the faeces and urine were collected separately and daily. The unchanged drug in the faeces and both free and water-soluble drug in the preserved urine were determined by the methods described and are expressed in Fig. 1. The total drug excreted in the urine was equivalent to 8.6% of 3074, of which 79% was in the water-soluble form. A similar experiment with a single 200-mg./kg. dose (one animal) gave values of 7.4% and 85% respectively. Three doses of 100 mg./kg. given on alternate days (14 animals) gave the equivalent of 23.5% of 3074 in the urine, of which 81% was metabolized.

Effects of Drug Administration on Normal Metabolites

(*a*) *Sulphur Metabolism.*—One rabbit (1.77 kg.) was fed on a diet of carrot and potato (100 g. of each daily) in order to minimize the output of ethereal sulphate, and the urine was analysed for inorganic and ester sulphate by the nephelometric method (Senior, 1947). After oxidation with Benedict's sulphur reagent (Benedict, 1909) the total sulphur was estimated by this technique, thus providing, by difference, organically combined sulphur other than ester sulphate. There was no significant alteration in the sulphur metabolism.

(*b*) *Glucuronide Excretion.*—Seven rabbits receiving the previously mentioned diet were each given a single 100-mg./kg. oral dose of 3074 and the daily output of glucuronides was estimated. The naphthoresorcinol method described by Hansen, Mills, and Williams (1944) was used with modifications (Senior, 1948). Care was taken to collect the urine in flasks containing mercuric chloride solution (1 ml. of 1% solution) in order to prevent the otherwise considerable losses of glucuronide due to fermentation and to avoid a false rise which would consequently appear if the voided drug or its metabolites were bacteriostatic (Senior, 1948). The mean value taken from the daily output during the four-day periods before and after dosing was equivalent to a rise of 10.6 mg. of glucuronic acid in a 2-kg. animal. This corresponds to 8% of the drug appearing in the urine as the glucuronide.

Photochemical Decomposition of 3074

It was observed that the standard solution of 3074 which contained 0.16% of acetic acid was quite stable to light, even after several months' exposure. The presence of alkali, however, resulted in a rapid disappearance of drug when the solution

was exposed to light through window glass. A series of solutions of the same strength (8 μg./ml.) but containing different concentrations of acid or alkali were exposed to daylight for 16 hours under identical conditions. Controls were kept in the dark. Four experiments with hydrochloric acid concentrations ranging between 0.025N and normality gave recoveries of 89–101% after exposure to light, and similar results of 92–105% were given by controls kept in the dark. A neutral solution gave values of 96% and 101% respectively. A similar range of solution containing sodium hydroxide gave complete recoveries when protected from light. On exposure, however, recoveries were 7.5% (0.025N solution) and 5.6% (0.1N). With stronger alkali the recoveries were higher, being 40% at 0.5N and 52% at normality. A second series of experiments was then performed under similar circumstances but employing a range of buffered solutions. The percentage recoveries, after exposure to light, were as follows: *p*H 6.8, 95; *p*H 7.0, 27; *p*H 7.2, 15; *p*H 7.4, 12.5; *p*H 7.6, 8.0; *p*H 7.8, 8.0; *p*H 8.0, 4.5%.

The investigation was extended to the biological fluids blood, plasma and urine, and 20% dilutions were used in lieu of the inorganic solutions. A solution of 8 μg./ml. of 3074 in a 20% plasma dilution was exposed to diffused sunlight for a period of twelve hours. There was a recovery of 21%, whereas a normal recovery was given by a similar solution kept in the dark. A medium of diluted urine again revealed photochemical decomposition, for a 55% recovery was obtained after exposure for twelve hours, whereas the value was 102.5% when protected from light. On the other hand, haemolysed blood gave a full recovery (97%) on exposure to light.

The possibility of this photochemical reaction resulting in the conversion of the drug to the water-soluble form was tested by exposing a solution containing approx. 8.5 μg./ml. in 0.1N-sodium hydroxide solution to sunlight (through glass) for two hours and then determining both free and total drug. The values recorded for free and total drug were 2.0 μg./ml. and 3.0 μg./ml. respectively. A control kept in the dark gave 8.7 μg./ml. and 8.4 μg./ml. respectively.

Biological Tests on the Reduced Form

The leuco- or dihydro-form of 6-aminoanthrapyrimidine was prepared by boiling the compound with aqueous alcoholic sodium hydrosulphite solution in the presence of ammonia (I. G. Farbenind, B.P. 461,883). The product $C_{15}H_{11}ON_3$ (Code No. 6262), m.p. 272°, gave only a slight fluorescence when shaken with benzene, gave no depression with 3074, and was re-oxidized at the melting point. It was of a lower solubility than the oxidized form, to which it rapidly reverted in solution. This reduced form has been reported to be slightly less active than the oxidized form, the minimal effective dose being 50 mg./kg. for 6262 compared with 30 mg. for the compound 3074 (Jones, Landquist, and Senior, 1952).

Extraction of 6262 with boiling alcohol followed by fluorimetric analysis of the filtrate revealed that less than 0.2% of unchanged 3074 was present. A single 100-mg./kg. oral dispersion administered to albino rats exposed to sunlight gave rise to an oedematous condition followed by necrosis, though the symptoms were rather less severe than those of a similar group of rats receiving the same dose of 3074. As this was very good evidence that there was an *in vivo* oxidation to the photodynamically active 3074, two similar groups of rats were given single 250-mg./kg. oral dispersions of 3074 and 6262, and the blood concentrations (Fig. 2) and liver concentration (Fig. 3) were

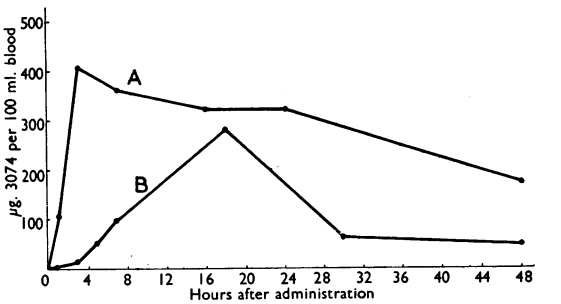

FIG. 2.—Blood concentrations of 3074 in rats following oral administration of dispersions of 3074 and 6262. Dose: 250 mg./kg. Three or four animals per point. A is 3074, B is 6262.

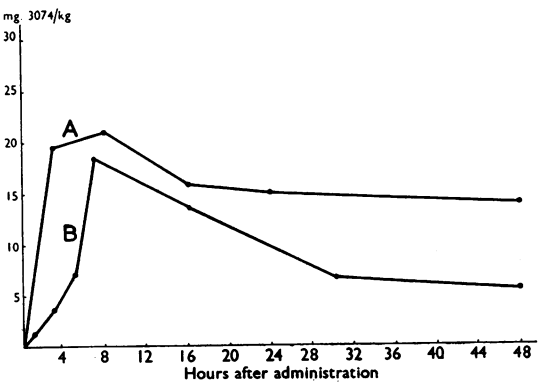

FIG. 3.—Liver concentrations of 3074 in rats following oral administration of dispersions of 3074 and 6262. Dose: 250 mg./kg. Three or four animals per point. A is 3074, B is 6262.

determined at suitable intervals, using three or four animals for each estimation. Other groups of six rats were given 100-mg./kg. doses of these substances, and the urine was analysed daily for both free and water-soluble drug for four days. The percentages of water-soluble metabolite to total drug excreted (free plus water-soluble) were 67 for 3074 and 85 for 6262.

Discussion

The substance 6-aminoanthrapyrimidine (3074) gave a high initial blood concentration in rabbit (100 mg./kg. oral), and this was maintained for at least 24 hours (Table I). Blood and liver concentrations in the rat were also of a high order (Figs. 2 and 3), and these persisted for at least 48 hours after a single 250-mg./kg. dose. Analysis of one rabbit killed 16 hours after one 100-mg./kg. oral dose revealed that 85% was absorbed (Table II), but only 4.3% of this could be accounted for. It was unfortunate that it was only in urine that it was possible to estimate the water-soluble form, for other tissues gave too high a blank reading on treatment with acid. The proportion of the water-soluble form in urine was approximately 80 to 85% of the total, and this value appeared to be approximately constant in all the samples of urine analysed. Even if the assumption be made that this ratio applies throughout the tissues the fate of the major portion of the drug would still be unknown.

It is clear that, although the dihydro- or leuco-form of the drug was not the main metabolite, it was probably the first stage in the degradation. There was no evidence that any other metabolite was present in the urine, and there was no significant rise in the excretion of the normal conjugating metabolites such as glucuronic acid or sulphur derivatives such as ester sulphate or mercapturic acid. Retention of the drug by protein binding is very unlikely. Though the anthrapyrimidine dyestuffs possess some affinity for natural fibres the 6-amino derivative is of low activity in this respect. This was reflected by the tissue recoveries, where the greatest loss due to protein binding was only 22.5% (lung), and was, of course, corrected for in the estimation. Analysis of the excreta over a period of several days confirmed that the drug was largely metabolized, for a single 100-mg./kg. dose gave rise to only 8.6% of total drug in the urine and 1.2% unchanged drug in the faeces. Likewise a 200-mg./kg. dose gave a total of 7.4% in the urine, though repeated dosing (three doses of 100 mg./kg.) gave a higher value of 23.5%.

The rise in glucuronides was probably not significant owing to the large variation in the normal excretion which occurred even on a controlled diet. The 100-mg./kg. dose was the highest which did not interfere with the food intake of the rabbit, and in view of the daily variation this was clearly too small to give a significant rise unless the major portion of the drug were to pass through to the urine and appear in the conjugated form. The observed rise in glucuronides suggested that 8% of the total drug administered was present in this form. It may have been a coincidence that this was of the same order as the finding from the fluorimetric analyses, for these were 8.6% of total drug and 6.8% of water-soluble drug. In any event the apparent rise may have been due to the excretion of other and undetected metabolites in the form of glucuronides, and it is generally not acceptable to claim that a metabolite appears as a glucuronide unless it has been isolated as such. The view is held, nevertheless, that the water-soluble form is solubilized by conjugation in some manner, probably as the glucuronide, and that it is hydrolysed on acidification, the resulting 6-aminodihydroanthrapyrimidine then undergoing aerial oxidation to 3074. Good evidence in favour of this was obtained from the synthetic 6-aminodihydroanthrapyrimidine (6262), which was only very sparingly soluble in water and which reverted to the oxidized form in solution.

The query arises as to which form of the drug was the active amoebicide. That the active substance was in no way associated with the photochemical decomposition has been clearly demonstrated by Jones (private communication), who showed that 3074 was equally curative in rats kept in the dark. Active metabolites were an improbability, for 3074 exhibited high *in vitro* activity (Jones, Landquist, and Senior, 1952), and yet this does not preclude the possibility that the drug may have previously been reduced by the medium. The synthetic reduced form 6262 was very nearly as active as 3074, but reference to Figs. 2 and 3 will reveal that the substance was re-oxidized on absorption and that there was consequently an appreciable blood and tissue concentration of 3074, though these values were considerably lower than those resulting from a similar dose of 3074. The two forms of the drug gave comparable urine analyses. The main evidence in favour of 3074 being the active amoebicide was that it was more active when given in this, rather than the reduced form, and that the blood concentration of the oxidized form was higher under these circumstances.

The photodynamic effect was obviously associated with the presence of the green fluorescent 3074 in the exposed parts. The appreciable blood concen-

tration illustrated its presence there, and *in vitro* tests revealed that the *p*H of the blood (*p*H 7.3–7.5) fell within the range required for photochemical decomposition. The compound was unaffected by alkali in the dark, was decomposed at *p*H 7 by light passing through window glass, and was stabilized by acid, for it was unaffected by light at *p*H 6.8. Complete recovery was obtained from diluted blood exposed to light, whereas in a medium of diluted plasma there was a considerable loss on exposure. This apparent anomaly is readily explained, for the intense coloration caused by the haemolysis would inevitably absorb most of the available light energy. The circumstances are very different *in vivo*, for the continual movement of the peripheral circulation ensures that the layers of blood or plasma are adequately exposed. Losses also occurred in urine on exposure to light, but these have no bearing on the accuracy of the determinations, for precautions were taken to collect the urine in shielded flasks.

The tissue reactions resulting in the oedematous condition and the photochemical decomposition of 3074 were thus shown to be taking place concurrently and in the same tissues. Whilst it is possible that the oedematous condition may be caused by the photochemical decomposition product, strong evidence against this is provided by naturally occurring fluorescent substances, or fluorescent metabolites of plant constituents, which have been observed in the blood of animals suffering from photogenic diseases such as hypericism, fagopyrism, and tribulosis (Brockmann, 1952). The active substances are of different chemical types and as a consequence bear no resemblance to 3074. There is little chance that this uncommon phenomenon would be caused by their unrelated photochemical decomposition products. Other synthetic fluorescent substances have long been known to exhibit this activity, and probably the first reported was eosin (Raab, 1903). Amongst medicinal compounds taken internally the fluorescent acriflavine (Jauson and Marceron, 1925) and riboflavin (Boehm, 1941) have given rise to photodynamic effects. As early as 1904 it was claimed that all substances which produce photodynamic action are fluorescent (Tappeiner and Jodlbauer, 1904), and no author has since demonstrated the phenomenon and at the same time proved the absence of a fluorescing substance from the affected parts.

Summary

1. A fluorimetric method has been devised for the estimation of 6-aminoanthrapyrimidine (3074) in animal tissue and excreta, and satisfactory recoveries have been obtained over the range of concentrations found in the rabbit and in the rat.

2. When administered orally to these animals as an aqueous dispersion, the drug was rapidly absorbed and gave a blood concentration ranging between 200 and 500 μg. per 100 ml., and this was maintained for at least 24 hours. The drug was rapidly metabolized, and only 1.5% of the original drug (100 mg./kg. dose) was detectable in the tissues of the rabbit after 16 hours.

3. Only a small proportion of the drug reappeared in the urine (2–4%), but a water-soluble form of the leuco-derivative was also present, and this was quantitatively reconverted to 3074 by treatment with dilute acid. The ratio of the two forms was roughly constant, and approximately 80% was in the reduced state.

4. Comparison of this water-soluble form with the synthetic leuco-derivative (6262) suggested that it was probably conjugated with a normal metabolite such as glucuronic acid. Oral administration of 6262 resulted in a partial oxidation to 3074 which was detectable in the blood, liver, and urine.

5. The photodynamic effect of 3074, in common with other synthetic and naturally occurring substances, was associated with its intense fluorescence, and the photochemical decomposition of the drug *in vitro* took place over the same *p*H range as that obtaining *in vivo*.

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