The Metabolism of Compounds Related to Ethanethiol

By G. A. SNOW
Imperial Chemical (Pharmaceuticals) Limited, Research Department, Blackley, Manchester 9

(*Received* 5 *June* 1956)

Several workers (Pianto, 1953; Brown *et al.* 1954; Kushner *et al.* 1955; Davies *et al.* 1956) have shown that ethanethiol and compounds closely related to it are strikingly effective antitubercular agents *in vivo* in several species of animal. The effect is confined to the ethanethiol series and is not shown by homologous thiols nor by any of the usual biological ethylating agents. It is noteworthy that *in vitro* the antitubercular activity of ethanethiol is small and non-specific, i.e. no greater than that of homologous thiols.

It seemed possible that the failure of ethanethiol to show activity *in vitro* might be explained by its conversion *in vivo* into a metabolite which was the active antitubercular agent. A metabolic study has therefore been carried out with, in the first instance, compounds related to ethanethiol labelled with ^{35}S. Diethyl disulphide, *S*-ethyl thiolbenzoate and *SS'*-diethyl dithiol*iso*phthalate were chosen for experiments on mice or guinea pigs, because these compounds were known to be potent antitubercular agents in the selected species.

EXPERIMENTAL

Preparation of compounds. Diethyl [^{35}S]disulphide was prepared by oxidation with solid I_2 of a solution of [^{35}S]-ethanethiol in 6N-NaOH, and ethyl [^{35}S]thiolbenzoate by benzoylation of a similar solution. Both compounds were purified by distillation. At the time of preparation, their activity was 31 mc/mole.

[^{35}S]Ethanethiol in NaOH was methylated with dimethyl sulphate; the resultant ethyl methyl [^{35}S]sulphide was dissolved in $CHCl_3$ and oxidized with NaOCl solution at pH 8. The ethyl methyl [^{35}S]sulphone was extracted and distilled; its activity at the time of preparation was 2·2 mc/mole.

SS'-Diethyl dithiol*iso*phthalate was prepared by the reaction of ethanethiol with *iso*phthaloyl chloride (Davies *et al.* 1956).

Measurement of radioactivity. Tissues were homogenized in water (usually 1 ml./g. of tissue) and the paste was spread evenly in polythene planchets 2·5 cm. in diameter. The planchets were placed immediately below the thin mica window of a Geiger–Müller tube and the count was recorded. Liquids were counted similarly. The amount taken was sufficient to give conditions of 'infinite thickness'. The results were recorded as counts/min. calculated for the undiluted tissue or liquid. The time of counting was sufficient to give a result of count corrected for background to an accuracy of 6% at 95% confidence limits. The methods of sample preparation were not thought to justify greater accuracy in counting.

In the experiments to be described, the amount of radioactivity administered is recorded in μc calculated from the initial activity of the source and the known rate of decay. Measurements are given in counts/min. calculated for the undiluted fluid or tissue. Under the conditions used, 1 μc/ml. of solution gave about 3000 counts/min.

Paper chromatogram strips 2 cm. in width were either cut into 2 cm. squares which were counted separately in planchets with an end-window tube and scaling unit, or the uncut strips were moved in 1 cm. steps past a tube having a mask pierced with a 2 cm. × 1·5 cm. rectangular opening (giving an overlap of 0·5 cm. for each section counted) and the counts recorded on a ratemeter. The maximum integrating time (140 sec.) of the ratemeter set a limit to the minimum amount of radioactivity detectable on the paper of about 8 counts/min. above background. With the scaling unit lower activities were detectable, but in practice the convenient length of time available for counting limited the minimum detectable level to about 3 counts/min.

Treatment of animals. Compounds for oral administration were prepared as 10% (w/v) dispersions in aqueous solution containing 1·5% of Dispersol OG and 1% of Cellofas WLD (both from Imperial Chemical Industries Ltd.), and were given by catheter. Compounds for subcutaneous injection were given as 25% (v/v) solutions in arachis oil. Animals were placed in metabolism cages and urine and faeces were collected separately. For some experiments mice were confined in a closed glass tank through which air was slowly drawn. The issuing air passed through traps containing permanganate solution.

Extracts of tissues. The tissue was homogenized with water (2 ml./g.) and the suspension was centrifuged. The residue was extracted twice more in a similar way leaving spent residue, *A*; the supernatants were pooled. Trichloroacetic acid (20%, w/v) was added (0·2 ml./ml. of extract) and the precipitate, *B*, removed by centrifuging. The solution was extracted three times with ether; the combined ether extracts, *C*, contained most of the excess of trichloroacetic acid. The aqueous solution, *D*, was concentrated under reduced pressure for paper chromatography. The fractions *A*, *B*, *C*, *D* contained 4, 9, 1 and 86% of the radioactivity respectively.

Concentration of organic metabolites from urine

By removal of urea and salts. Jack-bean meal (1·25 g.) was shaken with a solution of ethanol (15 ml.) and water (35 ml.) for 30 min., and then centrifuged. The supernatant was used as a urease preparation. Urine (100 ml.) from guinea pigs dosed with diethyl [^{35}S]disulphide was warmed to 30°; 5 mg. of KCN was added. Urease solution (50 ml.) at 30° was run in and the mixture was kept stirred; 5N-HCl was

run in as required to keep the pH between 6·3 and 7·3. After 2–3 hr. at 30°, change of pH ceased (acid added was approx. 8 ml.). The solution was acidified and N_2 was passed through to remove CO_2. Van Slyke determinations showed that 98% of the urea had been removed.

The solution was passed through a column containing a mixture of anionic and cationic resins [Zeo-Karb 225 (H), 40 g., and Deacidite FF (OH), 50 g.] to remove the electrolytes. The resultant solution was concentrated under reduced pressure for paper chromatography or countercurrent separation. Of the original radioactivity, 16% was recovered in the solution from the ion-exchange column; a similar activity was found in a sample of the same urine from which sulphate had been removed by addition of Ba^{2+}. Tests on the concentrate showed the metabolic product to be neutral (not absorbed by strong anionic or cationic resins), somewhat volatile and fairly polar. It was only very slightly extractable by ether and its partition coefficient in butanol–water was 0·48. It was stable to acids and alkalis.

A similar method of concentration was used for 20 l. of urine from guinea pigs dosed with inactive diethyl disulphide. In view of the volatility of the metabolite revealed by the experiments with radioactive material, the aqueous concentrate was evaporated to dryness under reduced pressure at the lowest practicable temperature, and the residue distilled. Crystallization of the distillate yielded slightly impure ethyl methyl sulphone; this was, however, more readily obtained by the solvent-extraction method.

By solvent extraction. Guinea pigs (approx. 500 g. body wt.) received a daily subcutaneous dose of *SS'*-diethyl dithio*iso*phthalate (200 mg./kg.) as a 50% (v/v) solution in arachis oil. This compound is well tolerated by the animals. Urine was collected and preserved by addition of $CHCl_3$. The combined urine (18 l.) was extracted with $CHCl_3$ (4 × 30 l.), the extract was filtered and the solvent distilled off. The residue was stirred with water (200 ml.) and ether (20 ml.). After separation, the aqueous layer was further extracted with ether (2 × 20 ml.). The combined ether layers on evaporation gave 2·5 g. of black waxy residue. The aqueous layer was extracted with $CHCl_3$ (5 × 200 ml.) and the solvent distilled off. The residue was distilled at 0·05 mm. giving fractions with b.p.'s 60–70° (0·50 g.), 70–105° (0·08 g.) and 105–120° (0·15 g.). Only a little non-volatile residue remained. The lowest-boiling fraction (m.p. 28°) was crystallized from methanol at −20°, giving 0·11 g. (m.p. 33·5°). Further crystallization from ether gave long colourless needles (0·08 g.), m.p. 33·5°, unchanged by admixture with ethyl methyl sulphone, m.p. 33·5°. [Beckmann (1878) reported 36°.] (Found: C, 33·0; H, 7·4; S, 29·5, 29·9. Calc. for $C_3H_8O_2S$: C, 33·3; H, 7·4; S, 29·7%.) No pure substance was isolated from the higher-boiling fractions.

Comparison of metabolites from urine with ethyl methyl sulphone

Ethyl methyl [^{35}S]sulphone was compared with crude urines from three species of animal dosed with diethyl [^{35}S]disulphide and with a purified concentrate of guinea-pig urine by paper chromatography in various solvents. The same concentrate was also examined by countercurrent distribution.

Paper chromatography. The downward method was used with Whatman no. 541 paper and the following solvents: (*a*) propan-1-ol, 280 ml., water, 250 ml., *n*-butanol to 1 l.; (*b*) $CHCl_3$, 200 ml., water, 200 ml., acetic acid to 1 l.; (*c*) 80% (v/v) dioxan in water. Aqueous phenol was unsuitable.

Countercurrent distribution. Ethyl methyl [^{35}S]sulphone (1 g.) was dissolved in water saturated with $CHCl_3$ and distributed through 49 transfers between $CHCl_3$ and water in a Craig machine (volume of upper and lower phases, 25 ml.). The contents of each tube were removed and the mixture was evaporated under reduced pressure until the $CHCl_3$ had been removed. The volume was then adjusted to 25 ml. with water and the aqueous solution was counted.

Urine concentrate (25 ml.) free from urea and electrolytes (activity, 425 counts/min. in 2·5 cm. planchet) was distributed exactly as for the ethyl methyl sulphone. The contents of each tube were concentrated, adjusted to 5 ml. and counted. Results appear in Fig. 4.

Examination of metabolite B. Solution from tubes 34–38 in the Craig separation described above containing metabolite *B* (see Fig. 4) was concentrated to 2 ml. Its behaviour on paper chromatography was compared with that of ethyl methyl sulphone and of ethyl methyl sulphoxide. The position of the (non-radioactive) sulphoxide spot was revealed by spraying with permanganate solution (Fig. 3).

The partition coefficient of ethyl methyl sulphoxide between $CHCl_3$ and water was estimated by equilibration and subsequent evaporation of the $CHCl_3$ layer and determination of the weight of residue. It was also estimated in both layers by oxidation with excess of permanganate and back titration of the excess. Neither method was very precise but the results were in rough agreement. Partition coefficient (K) by weight was 4·9, and by permanganate titration 4·6.

RESULTS

Preliminary experiments with mice showed that the labelled compounds were rapidly absorbed after oral or subcutaneous administration, and distributed evenly throughout the body tissues without any notable local concentration; a typical result is shown in Table 1. Excretion of radioactivity occurred mainly via the kidney. Faecal excretion was small, but appreciable excretion (14% of the dose in one experiment) occurred in the breath, probably as ethanethiol. In view of the volatile

Table 1. *Radioactive counts of tissues from mice receiving diethyl [^{35}S]disulphide*

Three 25 g. mice each received subcutaneously 10 mg. (2 μc) of diethyl[^{35}S]disulphide followed after 18 hr. by an equal dose; tissues were taken 4·5 hr. after the second dose.

Tissue	Combined wt. of organs (g.)	Radioactivity (counts/min.)
Alimentary tract (with contents)	12·6	183
Liver	4·69	126
Kidney	1·54	124
Spleen	0·57	92
Heart	0·50	94
Lung	0·82	128
Blood	1·3	118

nature of the thiol it was difficult to obtain accurate balances of radioactivity administered and recovered, but over a period of 24 hr. the urine of both mice and guinea pigs yielded 40–65 % of the radioactivity dosed in the form of relatively non-volatile compounds together with an undetermined amount of ethanethiol.

The course of urinary excretion of radioactivity in the guinea pig is shown in Fig. 1. A logarithmic plot of the fraction of the total excreted radioactivity against time gave a linear curve which was identical for diethyl disulphide and for ethyl thiolbenzoate. This result suggests that rate of excretion is governed by a single process; there is no evidence of rapid excretion of one product followed by slower excretion of another. In excretion of ^{35}S the two thiol-generating compounds apparently behaved similarly. Half the total ^{35}S was excreted in about 10 hr.

The nature of the radioactive compounds in mouse tissues was investigated by aqueous extraction and precipitation of protein. Most of the radioactivity remained in the aqueous solution after removal of the excess trichloroacetic acid by ether extraction. The nature of radioactive material taken down with the protein precipitate or not extracted from tissues by aqueous extraction (10–15 % of the total) was not determined. Other extraction procedures suggested that part of it was extractable into ether from aqueous suspension. The aqueous extract representing the major part of the relatively non-volatile radioactive material of the tissues was concentrated and examined by paper chromatography. At least 80 % of the radioactivity from muscle and visceral extracts, excluding the intestines, was found in a single peak in several different solvent systems (Fig. 2). A similar peak was observed with intestinal extracts, though considerable radioactivity remained at the origin.

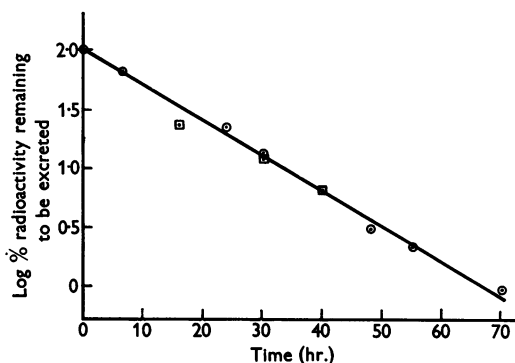


Fig. 1. Urinary excretion of radioactivity from a guinea pig (540 g.) dosed orally with ^{35}S-labelled compounds. ⊙, Ethyl thiolbenzoate, dose 200 mg. (27 μc); ⊡, diethyl disulphide, dose 100 mg. (40 μc).

When mouse urine was examined, a high activity was found at the origin and most of the remainder in a single peak identical with that found in the tissues (Fig. 2). The material which did not migrate in paper chromatography was precipitated from acid solution by Ba^{2+} ions and was identified as sulphate. Urine from guinea pigs dosed with labelled derivatives of ethanethiol resembled that from mice when examined in the same way, but rabbit urine showed two distinct and nearly equal peaks of radioactivity apart from the usual high level of sulphate.

The isolation and identification of ethyl methyl sulphone from urines of guinea pigs dosed with either diethyl disulphide (labelled and unlabelled) or diethyl dithio*iso*phthalate has been described above. It remained to establish the fact that ethyl methyl sulphone was the metabolite revealed by radioactive labelling. Radioactive sulphone was

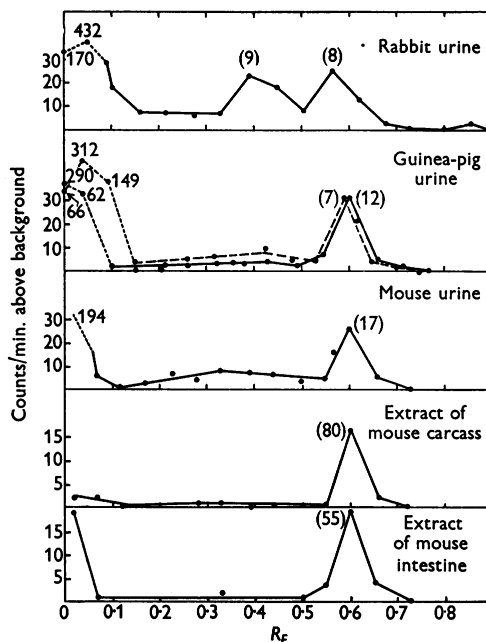


Fig. 2. Paper chromatography of urine and tissues from animals dosed orally with ^{35}S-labelled compounds. Rabbit (1·5 kg.); received 300 mg. (120 μc) of diethyl disulphide; 20 hr. urine. Guinea pig: ●—●, diethyl disulphide, 20 hr. urine; ●- - -●, ethyl thiolbenzoate, 6·5 hr. urine; wt. and dose as in Fig. 1. Mice (25 g.): each received 4 mg. (2 μc) of diethyl disulphide; urine and tissues were taken 4·5 hr. after dose. Amounts applied to paper: urine, 10 μl.; tissues, extract equivalent to 100 μg. moist wt. Developing solvent, butanol–propanol–water. Figures in parentheses show approx. percentage of total radioactivity in the peak. Dotted parts of curve are not to scale; counts/min. are shown by figures. All counts were made by scaler on sections of paper.

compared with radioactive concentrates of urine by paper chromatography and countercurrent distribution. The materials in three different solvent systems gave spots almost identical in position, except that those from the urine concentrate appeared to be rather more spread (Fig. 3). Distribution of labelled ethyl methyl sulphone between chloroform and water in a 50-tube Craig machine gave a single peak agreeing well with the theoretical distribution of a substance having K 0·69. The urine concentrate

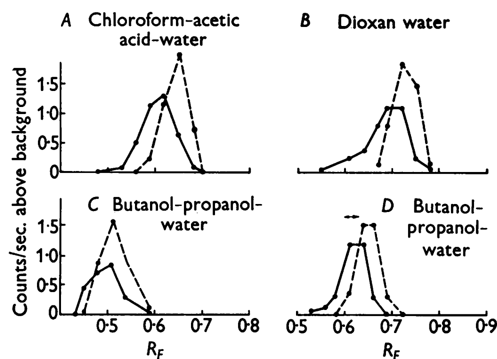


Fig. 3. Paper chromatography of ^{35}S-labelled metabolites of urine. Curves A, B, C: ●—●, guinea-pig urine after removal of urea and electrolytes; ●---●, ethyl methyl sulphone, 150 μg. (0·003 μc); developing solvents as indicated. Curve D: ●—●, metabolite B from countercurrent separation; ●---●, sulphone as in curves A, B, C; ↔, position of ethyl methyl sulphoxide spot. All counts were made on strips with ratemeter.

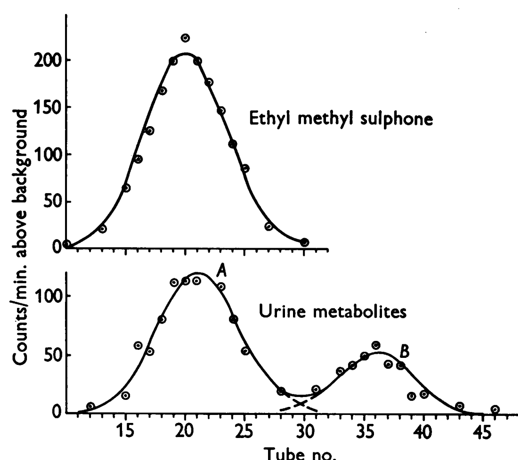


Fig. 4. Countercurrent distribution of ^{35}S-labelled metabolites of urine. Each separation involved 49 transfers between $CHCl_3$ and water. Points represent measured radioactivity of concentrates from individual tubes; curves are theoretical distributions: upper curve, K 0·69; lower curve, mixture of solutes, K 0·75 (1 part) and K 2·70 (0·4 part).

showed a very similar peak together with a second smaller one. The distribution of radioactivity could be well represented by the summation of theoretical curves for two substances having K 0·75 and 2·70 (Fig. 4). The slight difference in partition coefficient between the first peak and that of ethyl methyl sulphone is not regarded as significant. It only represents a difference of one tube in the series and may well be caused by the slight emulsification which occurred in separating the urine concentrate. The results indicate that ethyl methyl sulphone is certainly the main organic metabolite of ethanethiol derivatives in urine. There is also a second metabolite, B, present to the extent of 40% of the sulphone.

Metabolite B is evidently not readily separable from the sulphone by paper chromatography in three quite diverse solvents. It is not certain whether it occurs in the tissues, since the only comparison between the metabolites in urine and tissues was made by paper chromatography. Its nature has not yet been established, and it is not known whether it is a constituent of the higher-boiling fraction obtained in the extraction of non-radioactive urine. Paper chromatography showed that ethyl methyl sulphoxide could just be separated from the sulphone, but could hardly be separated from metabolite B (Fig. 3). However, approximate determinations of the partition coefficient of the sulphoxide between chloroform and water gave values considerably different from those found for metabolite B in the countercurrent separation. The question of the identity of this product thus remains unsolved.

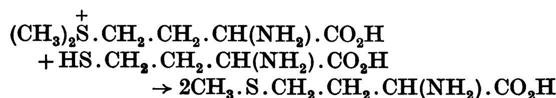
Neither ethyl methyl sulphone nor a crude mixture containing metabolite B was significantly effective in protecting mice from tubercular infection.

DISCUSSION

Few investigations have been made of the metabolism of alkyl thiols. Canellakis & Tarver (1953) showed that when methanethiol is given intraperitoneally to rats, the sulphur appears as sulphate in the urine, and is not significantly incorporated into the methionine or cysteine of liver protein. The carbon atom is available for incorporation into the methyl groups of choline and methionine and into the β-carbon atom of serine. According to the present experiments with three animal species the sulphur of ethanethiol is excreted mainly as inorganic sulphate. However, organic metabolites account for 10–20% of the sulphur excreted, and the chief of these in the mouse and guinea pig is ethyl methyl sulphone. In tissues the sulphone preponderates, probably because of a more rapid excretion of sulphate; it is also possible that the breakdown to sulphate occurs in the kidney itself.

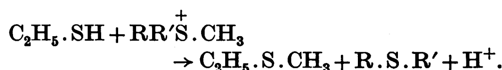
The most probable course of the methylation and oxidation involved in the conversion of ethanethiol into ethyl methyl sulphone would appear to be a preliminary conversion into ethyl methyl sulphide, followed by oxidation to the sulphone. Evidence for biological methylation of sulphur compounds related to ethanethiol has been presented by Challenger & Rawlings (1937), who showed that the mould *Scopulariopsis brevicaulis* converts diethyl disulphide into ethanethiol and ethyl methyl sulphide. At least one example of the oxidation of a sulphide to a sulphone *in vivo* has been reported. Rose & Spinks (1948) showed that *p*-methylthiolaniline was readily converted into the corresponding sulphone by mice, rats or rabbits.

Schlenk & Palma (1955) proposed the reaction



$$(CH_3)_2\overset{+}{S}.CH_2.CH_2.CH(NH_2).CO_2H + HS.CH_2.CH_2.CH(NH_2).CO_2H \rightarrow 2CH_3.S.CH_2.CH_2.CH(NH_2).CO_2H$$

to explain the observation that a mixture of homocysteine and methionine *S*-methiodide can replace methionine as a source of methylthioadenosine in yeast, although neither is nearly as effective separately. A similar type of reaction with a sulphonium compound could be imagined as a mechanism for the methylation of ethanethiol:



$$C_2H_5.SH + RR'\overset{+}{S}.CH_3 \rightarrow C_2H_5.S.CH_3 + R.S.R' + H^+.$$

Sulphonium compounds occur in living tissues, and one, *S*-adenosylmethionine, has already been indicated as an intermediate in biological methylation (Cantoni, 1953); the methyl sulphonium compound corresponding to methionine has been found in plant tissues (Challenger & Hayward, 1954; McRorie *et al.* 1954). Another way in which alkyl sulphides can arise by an enzymic process is illustrated by their formation in a lichen preparation from sulphonium compounds of a thetin type (Challenger & Liu, 1950). It would, however, seem preferable not to postulate the formation of an ethyl sulphonium compound of the type $C_2H_5.\overset{+}{S}(CH_3)R$ since it is doubtful whether the radioactive label would be retained in the formation of such a derivative, and, further, the intermediate might be expected to function like ethionine as a biological ethylating agent. No evidence has ever been observed of the ethionine type of toxicity (Stekol & Weiss, 1949) when compounds related to ethanethiol have been administered in frequent high doses to mice or guinea pigs over periods of several weeks. It is, however, not certain how sensitive these species may be to interference with the normal methyl metabolism.

It seems improbable that the carbon of ethanethiol can be available for incorporation into compounds in the same way as that of methanethiol (Canellakis & Tarver, 1953), but knowledge of the fate of carbon atoms released in the formation of sulphate must await the result of experiments with carbon-labelled material. It is not known whether sulphones occur in the metabolism of the normal sulphur compounds of the body, but Ruzicka, Goldberg & Meister (1940) isolated dimethyl sulphone from ox blood. Experience suggests that such chemically inert and rather volatile compounds may easily be overlooked in metabolic studies.

The features of ethanethiol metabolism revealed in these experiments provide no explanation of its specific antitubercular action *in vivo*, since neither ethyl methyl sulphone nor the unidentified metabolite shows this activity. The possibility remains that some minor metabolite, not revealed by the separation methods used, could be the effective agent. However, almost every possible oxidative metabolite and derivative of ethanethiol has been prepared and tested (Davies *et al.* 1956), and none was active unless easily convertible into the thiol. Present evidence favours the view that the antitubercular activity is due to ethanethiol itself. This compound is certainly very quickly removed from circulation by metabolism, and the greater relative effectiveness of certain derivatives of the thiol (notably the thiol esters), especially by subcutaneous injection, probably reflects a comparatively slow release of the parent substance. It is known that early multiplication of the tubercle bacillus occurs largely in the macrophages, and the specific inhibitory action of ethanethiol may result from some peculiarity of local metabolism in the macrophage or in the micro-organism growing in this special environment.

SUMMARY

1. Diethyl disulphide and *S*-ethyl thiolbenzoate (labelled with ^{35}S) have been given to mice and guinea pigs and the radioactivity of tissues and excreta measured. Radioactivity is rapidly and fairly uniformly distributed throughout the body.

2. Radioactivity is excreted mainly in the urine; in a guinea pig 50 % of the total urinary excretion occurred in about 10 hr. Little radioactivity is excreted in the faeces, but a substantial amount in the breath in the form of ethanethiol.

3. Sulphate accounts for 80–90 % of the radioactivity in urine. Two organic metabolites are also present, one of which is ethyl methyl sulphone; the other has not been identified.

4. The major part of the radioactivity found in the body tissues of dosed mice comes from ethyl methyl sulphone, perhaps in association with the unidentified organic metabolite.

5. Neither ethyl methyl sulphone nor a crude mixture containing both organic metabolites showed any marked antitubercular activity when tested *in vivo*.

The author is indebted to Mr G. E. Davies and Dr A. R. Martin for dosing and care of animals, and to Mr J. C. B. Smith for technical assistance. Dr G. W. Driver kindly provided the *SS'*-diethyldithiol*iso*phthalate.

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Ion Transport and Respiration of Isolated Frog Skin

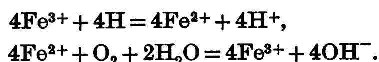
By A. LEAF* and A. RENSHAW

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford, and The Howard Hughes Medical Institute

(*Received* 11 *June* 1956)

In the search for the mechanism of active ion transport by cells the hypothesis was early introduced that such transport might be causally linked to the transference of electrons in the oxidation–reduction reactions of the cells. The concept of oxidation–reduction reactions as sources of bioelectric phenomena in cells and tissues was first introduced by Lund in 1928. Ten years later this concept was applied to the movement of ions and secretory processes by Friedenwald & Stiehler (1938) and Stiehler & Flexner (1938). Subsequently, this hypothesis was revised and extended by a number of workers studying ion transport (Lundegårdh, 1939; Robertson & Wilkins, 1948*a*, *b*; Conway & Brady, 1948; Crane & Davies, 1948; Patterson & Stetten, 1949; Rehm, 1950). These views have been incorporated by Davies & Ogston (1950) into their mechanism I and by Conway (1951, 1952, 1953, 1955) who has conveniently termed this hypothesis the 'redox pump' theory of ion transport.

Basically these hypotheses state that the following two reactions occurring in aerobic metabolism are spatially separated within the cell:



$$4Fe^{3+} + 4H = 4Fe^{2+} + 4H^{+},$$
$$4Fe^{2+} + O_2 + 2H_2O = 4Fe^{3+} + 4OH^{-}.$$

Fe^{3+} and Fe^{2+} are the oxidized and reduced forms of cytochrome iron; hydrogen atoms arise from the substrate of metabolism. The electrons picked up from the hydrogen atoms in the first reaction are considered to be passed along an oxidation–reduction chain to another site in the cell where they are finally received by molecular oxygen. The hydrogen ion formed in these reactions would be available for secretion as such, or for exchange with cations outside the cell. The same reasoning can be applied to make this system transport anions. Oxidation–reduction systems which do not contain iron have been similarly considered (Patterson & Stetten, 1949).

As pointed out by Lund & Stapp (1947) and Robertson & Wilkins (1948*b*), this hypothesis has a quantitative limitation which may be tested experimentally. A maximum of four electrons, or

* Investigator, Howard Hughes Medical Institute. Permanent address: Department of Medicine, Massachusetts General Hospital, Boston, Mass., U.S.A.