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# Uptake of antimony potassium tartrate by mouse liver slices

S. E. SMITH

*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.1*

1. The uptake of $^{124}$Sb-antimony potassium tartrate by isolated mouse liver slices has been measured and found to establish high tissue: medium concentration ratios.

2. Uptake was not influenced by oxygen lack, potassium, ouabain, dinitrophenol or sodium arsenate. It was inhibited by dimercaprol and reduced at low temperature. No evidence was found of counter-transport. After subcellular fractionation, most of the radioactivity was recovered from particulate fractions.

3. Kinetic studies of uptake from media containing different concentrations of antimony suggest that uptake is due partly to diffusion and partly to a saturable binding mechanism, probably involving chelation by non-diffusible thiol groups. Saturation studies suggest that only a small proportion of thiol groups bind antimony, the remainder undergoing catalytic oxidation.

It is well established that trivalent antimony compounds given to animals and man are taken up and stored by the liver (Brady, Lawton, Cowie, Andrews, Ness & Ogden, 1945; Gellhorn, Tupikova & Van Dyke, 1946; Abdallah & Saif, 1962). Recent work has suggested that, though the mechanisms involved are obscure, entry of antimony into the liver may be dependent on an active transport system (Rowland, 1968). If this is so, it is possible that in clinical practice the treatment of schistosomiasis with antimonials might be improved by deliberate antagonism of the liver uptake without affecting schistosomal uptake. The present work on antimony uptake by liver slices *in vitro* was undertaken with this in mind.

## Methods

### *Uptake experiments*

Male white mice were killed by decapitation. The left anterior lobe of the liver was sliced transversely with a mechanical chopper (McIlwain & Buddle, 1953) and the slices (thickness 312$\mu$) suspended in a Krebs-Ringer medium of the following composition: NaCl, 138 mM; KCl, 4·8 mM; $CaCl_2$, 2·6 mM; $MgSO_4$, 1·2 mM; $Na_2HPO_4$-$NaH_2PO_4$ buffer (pH 7·4), 3·9 mM; glucose 11·1 mM. The concentration of buffer used, one quarter of the standard amount, was found to be the greatest

which permitted stability of $Ca^{2+}$ in solution (Smith, 1967). Two to four slices (weight 30–80 mg) were placed in conical flasks (50 ml.) in 14·5 ml. medium, gassed with oxygen and preincubated at 37° C for 10 min. Following this, 0·5 ml. quantities of $^{124}$Sb-labelled antimony potassium tartrate solution (Radiochemical Centre: batches 513901, 525796 and 562062) were added. Each addition was diluted with unlabelled antimony potassium tartrate to bring the overall concentration in the medium to $7{\cdot}7 - 250 \times 10^{-6}$M. After incubation for various times, slices were removed, blotted dry, weighed, digested in $H_2SO_4$ and counted for radioactivity in a well scintillation counter. Tissue antimony concentrations were expressed as mμ-moles/g wet weight or as tissue:medium concentration ratios.

The capacity for binding antimony was measured in two ways: first by incubating slices to equilibrium at 2, 3 or 4 hr as above, second by 24 hr dialysis of homogenates in ¼ inch Visking tubing bags against 0·01 M phosphate buffer pH 7·4. In each case labelled antimony potassium tartrate of various concentrations was added to the medium and the final tissue and medium concentrations measured. The results were combined and plotted as reciprocals of bound against free antimony and a suitable regression line fitted by eye. A standard linear regression calculation is not strictly appropriate because of the reciprocal data and the errors present on both axes. The binding capacity of the tissue was obtained by extrapolation.

In some experiments, slices of mouse brain (cerebral hemisphere), kidney, intestine (jejunum) and spleen were treated as for liver and incubated for 60 min in the presence of antimony potassium tartrate $10^{-5}$M.

In one experiment, slices of liver, cut as above, were incubated in medium containing $^{14}$C-inulin (New England Nuclear Corporation: specific activity 3·08 μc/mg). The slices were then blotted dry, weighed, homogenized in 5% trichloroacetic acid and centrifuged. Supernatants were counted in a Beckman liquid scintillation spectrometer using a toluene:Triton X-100 base scintillator (Patterson & Greene, 1965). Inulin space was calculated as the tissue:medium ratio.

### *Subcellular fractionation*

In five experiments, slices incubated for 60 min in the presence of $^{124}$Sb-antimony potassium tartrate $10^{-5}$M were homogenized with a Teflon and glass homogenizer in 0·32 M sucrose containing 0·5 mM EDTA.$Na_2$ neutralized to pH 7·4 with Tris. The homogenate was differentially centrifuged at 4° C to produce nuclear, mitochondrial, microsomal and supernatant fractions (Hebb & Whittaker, 1958). Individual fractions were digested and counted as above. Protein was assayed colorimetrically (Folin & Ciocalteu, 1927).

### *Liver slice respiration*

Oxygen uptakes of liver slices, cut as above and suspended in the same medium, were measured by Warburg manometry at 37° C. Antimony potassium tartrate was incorporated in the medium to final concentrations of $10^{-5}$ to $10^{-3}$M.

### *Thiol group estimation*

Free thiol groups of liver and other tissues were estimated spectrophotometrically (Jocelyn, 1962), using 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent).

## Results

Liver slices accumulated antimony establishing a concentration gradient. The time course of net uptake at two different concentrations in the medium is shown in Fig. 1. At both concentrations net uptake increased with prolonged incubation but was not linear with time. After 2 hr incubation at a concentration of $10^{-5}$M, tissue: medium ratios rose to a mean of 38·3:1, indicating very powerful concentrating ability. No further uptake occurred after 2 hr at any concentration. Antimony contents at 2, 3 and 4 hr were combined with values obtained from dialysis of liver homogenates to provide an estimate of total binding capacity. A reciprocal plot of binding against concentration in the medium (Fig. 2) yielded a theoretical maximum

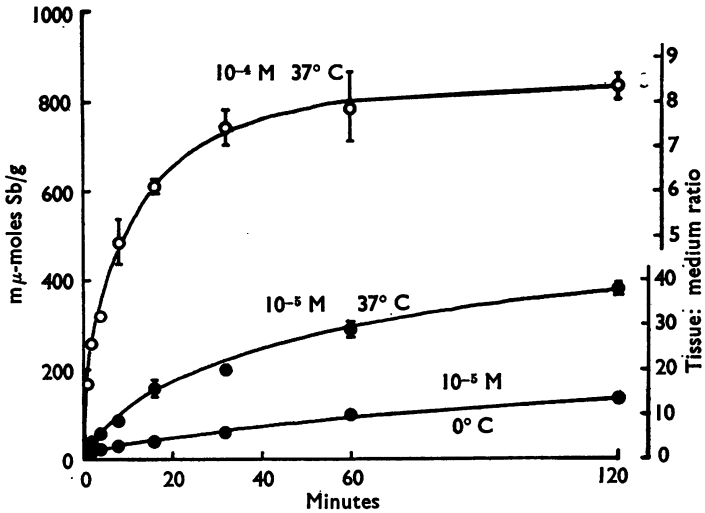


FIG. 1. Time course of uptake of $^{124}$Sb-antimony potassium tartrate by mouse liver slices. Each vertical line represents S.E. of mean. Initial concentrations of Sb in medium indicated.

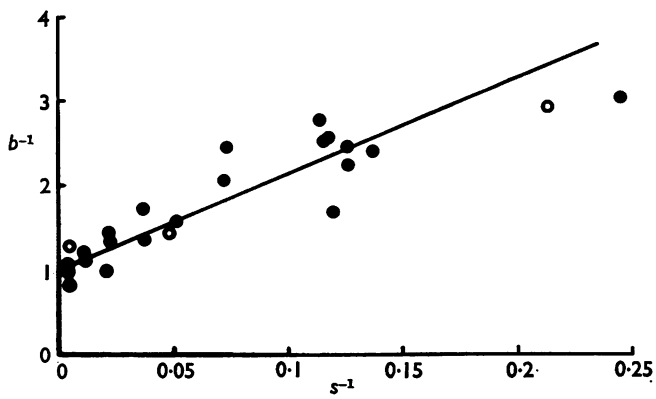


FIG. 2. Reciprocal plot of equilibrium binding of $^{124}$Sb by mouse liver slices. *s*, Equilibrium concentration of Sb in medium (μM). *b*, Concentration of Sb in tissue (μ-moles/g). Filled circles, liver slices; unfilled circles, liver homogenates. Line fitted by eye (see **Methods**).

binding capacity of about 1·0 $\mu$-mole/g. At 0° C net uptake was reduced by about 70% though incubation for 2 hr still established the high concentration ratio of 13·5 : 1. The comparable ratio obtained with inulin was 0·44 : 1. Concentrations of antimony in the medium never fell by more than 9·1%.

With prolonged incubation the liver slices obviously deteriorated, strands of white dead-looking tissue separating in the incubation medium. These were routinely discarded but on six occasions after 2 hr incubation were counted and found to have taken up more antimony weight for weight than the healthy tissue. Four samples obtained at 37° C contained 3 to 4 times and two samples obtained at 0° C contained 16 to 18 times as much as the healthy tissue. Microscopic examination of similar samples revealed only undifferentiated necrotic tissue.

Initial rates of uptake (1 min) from media containing six concentrations of antimony in the range 7·7 to $250 \times 10^{-6}$M were measured. The results are illustrated as a reciprocal plot of velocity against substrate concentration (Fig. 3) as described by Lineweaver & Burk (1934). Linear regression analysis of these results provided a poor fit ($\chi^2 = 7{\cdot}914$; $0{\cdot}2 > P > 0{\cdot}1$) suggesting that more than one component was involved in the uptake process. The results were analyzed also on the assumption that uptake was made up of two components, a saturable Langmuir function and a diffusional one (Smith, 1967). The line drawn is the calculated best fit ($\chi^2 = 1{\cdot}8002$; $0{\cdot}9 > P > 0{\cdot}8$) which yields: maximum transport velocity ($V_m$) = 141·3 m$\mu$-moles $g^{-1}$ $min^{-1}$, transport constant ($K_t$) = $59{\cdot}2 \times 10^{-6}$M and diffusion constant ($K_d$) = 0·84 ml. $g^{-1}$ $min^{-1}$.

After subcellular fractionation of liver slices incubated in $^{124}$Sb-antimony potassium tartrate $10^{-5}$M for 60 min, approximately 70% of the radioactivity was recovered from particulate fractions (Fig. 4). The microsomal fraction contained the highest concentration in terms of both protein and tissue weight, though the total radioactivity contained in the fraction was small. The mean concentration ratios

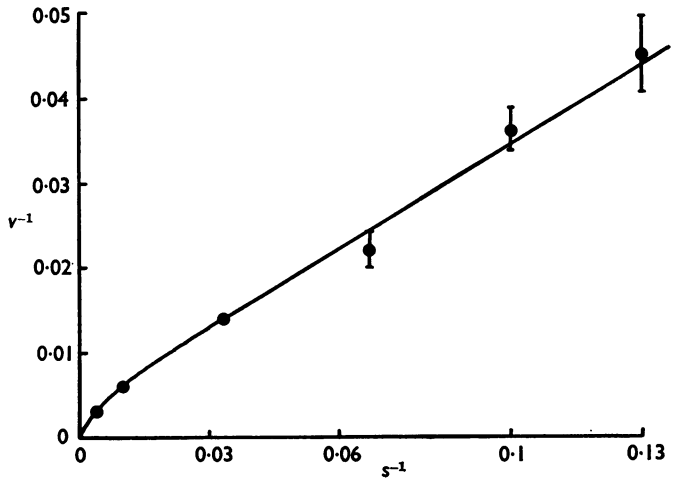


FIG. 3. Reciprocal plot of 1 min uptake of $^{124}$Sb-antimony potassium tartrate by mouse liver slices (twenty-five measurements). Each vertical line represents S.E. of mean. *s*, Initial concentration of Sb in medium (m$\mu$-moles/ml.). *v*, Uptake velocity (m$\mu$-moles/g per min). (Mean ± S.E.) Line drawn is best fit as described in text.

TABLE 1. *Uptake of $^{124}$Sb-antimony potassium tartrate (APT) by mouse liver slices. Effect of oxygen lack, potassium, ouabain, dinitrophenol (DNP), dimercaprol, dithiothreitol, Ellman's reagent and sodium arsenate*

| | $^{124}$Sb-APT uptake (mμ-moles/g) | | | |
|---|---|---|---|---|
| | 1 min | 2 min | 4 min | 8 min |
| Control (mean) | 26·7 | 39·3 | 55·8 | 85·0 |
| 95% confidence limits | 23·0–30·3 | 34·3–44·2 | 45·1–66·4 | 76·1–93·9 |
| Oxygen lack | 23·7 | 37·6 | 55·8 | 81·3 |
| | 24·8 | 35·0 | 56·6 | 75·4 |
| DNP $10^{-4}$M | 26·2 | 41·9 | 60·8 | 89·6 |
| | 25·5 | 47·2 | 57·8 | 71·9 |
| KCl 40 mM | 22·9 | 36·0 | 52·6 | 75·4 |
| Ouabain $10^{-5}$M | 21·4 | 35·4 | 54·7 | 91·6 |
| | 27·5 | 35·7 | 53·5 | 78·0 |
| Dimercaprol $1{\cdot}24 \times 10^{-4}$M | 15·6 | 17·4 | 22·4 | 26·9 |
| Dithiothreitol $10^{-3}$M | 20·5 | 28·5 | 44·2 | 63·2 |
| | 22·9 | 34·0 | 51·0 | 76·6 |
| Ellman's reagent $10^{-4}$M | 13·8 | 22·1 | 35·7 | 61·7 |
| | 15·1 | 21·6 | 36·4 | 62·9 |
| Na arsenate $10^{-3}$M | 28·3 | 40·0 | 60·4 | 90·9* |
| | 25·4 | 36·6 | 52·9 | 78·8† |

Concentration of APT in medium = $10^{-5}$M.
* Na arsenate preincubated for 1 hr.
† Na arsenate added without pre-incubation.
All other additions preincubated for 10 min.

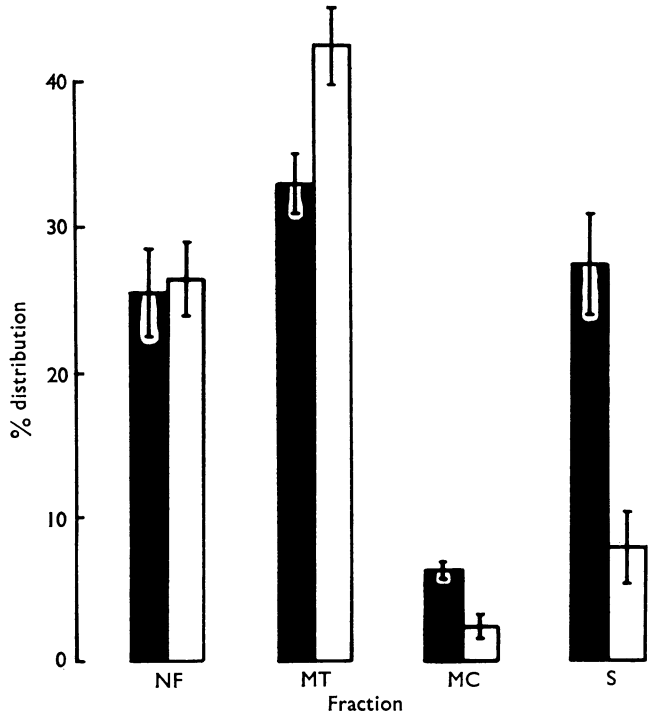


FIG. 4. Percentage distribution of radioactivity and protein in subcellular fractions of mouse liver slices incubated with $^{124}$Sb-antimony potassium tartrate 10 mμ-moles/ml. for 60 min. Filled columns: radioactivity; unfilled columns: protein. Vertical lines represent S.E. of mean. NF, Nuclear fraction; MT, mitochondrial fraction; MC, microsomal fraction; S, supernatant.

for mitochondrial fraction: supernatant and microsomal fraction: supernatant were 37·6:1 and 47·0:1 respectively.

Slices of other tissues were able to take up antimony though they did so less than liver slices (Fig. 5). Accumulating ability decreased in order: liver>kidney> spleen>intestine>brain.

Antimony uptake by liver slices incubated for 1 to 8 min was unaffected by the absence of oxygen and by the presence of KCl 40 mM, ouabain $10^{-5}$M or dinitrophenol $10^{-4}$M added to the medium 10 min beforehand. Sodium arsenate $10^{-3}$M added 1 hr earlier or at the same time as the antimony also had no effect. Dimercaprol $1{\cdot}24 \times 10^{-4}$M inhibited uptake, as did also dithiothreitol $10^{-3}$M and Ellman's

TABLE 2. *One minute uptakes of $^{124}$Sb-antimony potassium tartrate (APT) by mouse liver slices pre-incubated for different times with non-labelled APT* ($10^{-5}$M)

| Preincubation time with APT (min) | 1 min $^{124}$Sb-APT uptake (mμ-moles/g) | Preincubation time with APT (min) | 1 min $^{124}$Sb-APT uptake (mμ-moles/g) |
|---|---|---|---|
| 0 | 26·7 | 8 | 21·9 |
| 95% confidence limits | 23·0–30·3 | | 26·2 |
| 1 | 28·2 | 16 | 26·6 |
| | | | 30·2 |
| 2 | 23·1 | 32 | 28·6 |
| | 24·1 | | 25·6 |
| 4 | 27·6 | 60 | 34·0 |
| | 30·9 | | 27·6 |

Concentration of APT in medium = $10^{-5}$M during preincubation and incubation periods.

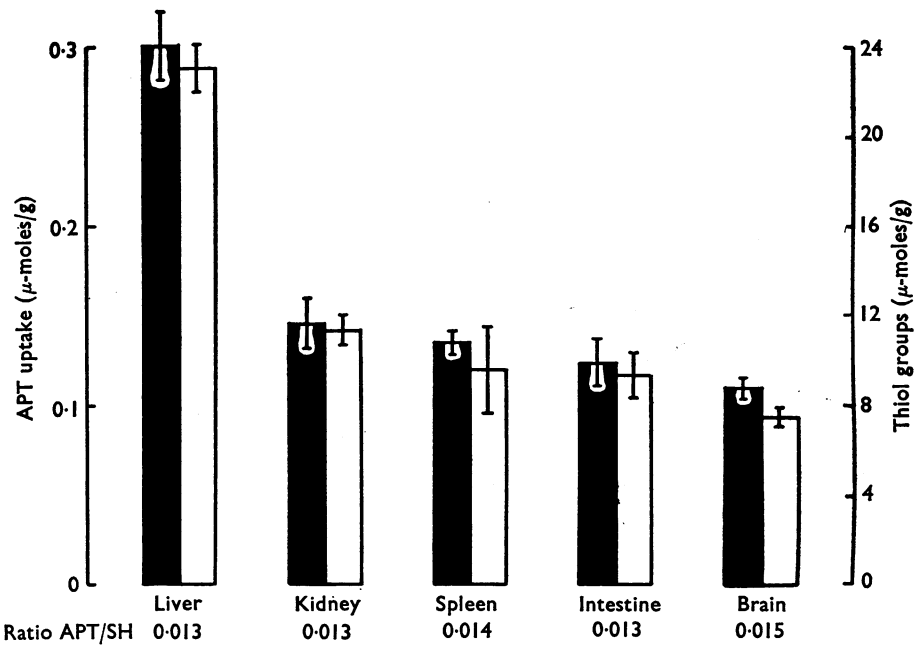


FIG. 5. Uptake of $^{124}$Sb-antimony potassium tartrate (APT) in 60 min (filled columns) by mouse tissue slices and thiol group content of untreated tissues (unfilled columns). Mean (±S.E. of mean) of six and five measurements respectively.

reagent $10^{-4}$M, though to a lesser extent. These results are shown in Table 1. Pre-incubation of the slices with unlabelled antimony potassium tartrate $10^{-5}$M for periods up to 60 min did not influence subsequent uptakes of the labelled compound (Table 2).

Fresh mouse tissues were assayed for their content of free thiol groups (Fig. 5). There appeared to be a relationship between the ability of the tissues to take up antimony and their thiol group content, though only a small fraction of the groups was involved. Fresh liver contained 23 $\mu$-moles thiol/g (Fig. 5). After incubation for 60 min liver slices contained less measurable thiol content (6·93 $\mu$-moles/g) and this was further reduced by incubation in the presence of antimony potassium tartrate at different concentrations (Table 3). This further reduction was greater than could be accounted for by the amount of antimony accumulated (Figs. 1 and 2). The uptake of antimony by liver slices was associated with progressive reductions in oxygen consumption (Fig. 6). At the highest concentration used ($10^{-3}$M), oxygen consumption was reduced by about 90% and the tissue slices became markedly decolorized.

TABLE 3. *Effect of antimony potassium tartrate (APT) on mouse liver slice thiol group content after incubation for 60 min*

| APT concentration | Thiol groups ($\mu$-moles/g) |
|---|---|
| 0 | 6·93±0·75 |
| $10^{-5}$M | 5·80±0·73 |
| $10^{-4}$M | 2·38±0·71 |
| $10^{-3}$M | 0·93±0·28 |

Figures represent means of five experiments ±S.E. of mean.

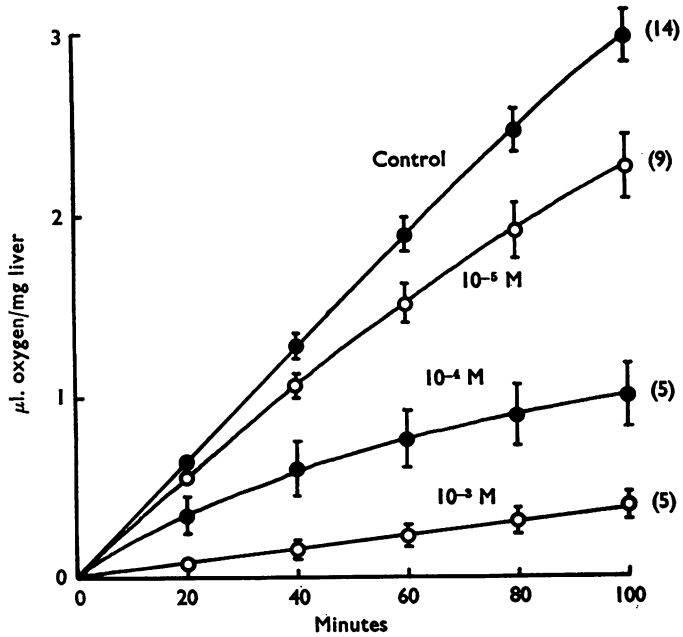


FIG. 6. Effect of antimony potassium tartrate on oxygen consumption (mean ±S.E. of mean) of mouse liver slices. Figures in parenthesis indicate the number of experiments. Concentrations of antimony in the medium indicated.

**Discussion**

The results reported here confirm that the liver can take up antimony and store it against a concentration gradient to an extent which can lead to high tissue levels. The findings that the uptake is only moderately reduced by cooling and is not influenced by oxygen lack nor the presence of dinitrophenol suggest that this process is not energy dependent. The lack of influences of potassium in high concentration and of ouabain suggests also that uptake is not linked to ion transport, nor is it likely to be carrier-mediated because prolonged incubation with the metal did not accelerate its rate of uptake. Systems which are carrier-operated usually show counter-transport. Movement of antimony across the cell membrane, if it does cross the cell membrane, therefore, appears to be due to diffusion.

The concentrating ability of the tissue must depend on some specific binding capacity either on the cell membrane or within the cell substance. The binding site has not been localized in the present experiments because the evidence provided by subcellular fractionation is not necessarily conclusive. The attachment of antimony to the particulate fractions could have occurred during the prolonged separation procedure involved. The site would best be identified by autoradiography but the available isotopes of the metal are all powerful gamma-emitters, making them unsuitable for this type of study. The site is most likely to involve chelation by protein thiol groups, an explanation which has been offered for the storage of other heavy metals by Ehrlich tumour cells (Pal & Christensen, 1959) and of ferric iron by rat liver slices (Saltman, Fiskin & Bellinger, 1956). Such an explanation is consistent with the high recoveries of the metal from subcellular particulate fractions and with some preliminary findings that human plasma protein binds antimony, a property which might be shared by tissue proteins. It is also consistent with the finding that part of the uptake conforms to Michaelis-Menten kinetics. It seems likely that the rate limitation is imposed by the binding mechanism rather than by the transport of the metal through cellular membranes.

If liver thiol groups bind antimony, the experiments using equilibrium conditions suggest that only a small proportion of these groups is involved: about 1 in 23 $\mu$-moles/g. Many of these groups are undoubtedly part of diffusible molecules for they disappear from the liver slices during incubation. There remain about 7 $\mu$-moles/g, all of which are potential chelators. The question arises as to why the tissue does not bind even more of the metal. The uptake of antimony is associated with a greater disappearance of thiol groups than can be accounted for by the amount of the metal accumulated. At the same time oxygen uptake by the tissue is largely abolished. It must be assumed that the remaining thiol groups undergo catalytic oxidation, thus limiting the chelation of the metal and the accumulating ability of the tissue. This, rather than chelation, may account for much of the toxicity of antimonials when used clinically (Saz & Bueding, 1966). It was expected that dithiothreitol might have a thiol-sparing effect and increase liver slice uptake of antimony. Its failure to do this suggests that this compound itself chelates the metal in solution to some extent and thus acts like a lower concentration of dimercaprol. The antagonistic effect of Ellman's reagent, used in other experiments to measure thiol groups, is presumably due to partial inactivation of the tissue thiol groups responsible for the binding. It is possible, however, that this compound may react with thiol groups in the cell membrane and thus interfere with the permeability of the membrane to the metal.

*In vivo* antimonials accumulate in the liver yet hardly at all in other organs. As shown here, slices of other tissues can concentrate the metal to a considerable extent, though less so than can liver slices. This ability appears to be proportional to their thiol group contents. The difference between *in vitro* and *in vivo* results is hard to explain unless one assumes that antimony cannot cross some barrier between the circulation and the tissue except in the liver. The identity of this barrier remains obscure. Part of the interest of this study lay in the possibility of finding conditions in which liver antimony uptake might be reduced when the metal is used clinically. In view of the binding mechanism which appears to be involved, this seems unlikely.

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