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The Effect of Trypan Blue, Suramin and Aurothiomalate on the Breakdown of ^{125}I-Labelled Albumin within Rat Liver Lysosomes

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1. A fraction enriched in lysosomes was prepared by centrifugation from the livers of rats that had been injected 0.5h before death with ^{125}I-labelled albumin. When suspended in sucrose-protected buffer, pH 7.4, and incubated at 22°C for 2h, the particles progressively released iodotyrosine into the medium. Albumin digestion did not occur if the particles were subjected to treatments known to break lysosomes or if particles from uninjected rats were incubated in medium containing ^{125}I-labelled albumin. It is concluded that the observed production of iodotyrosine results from protein hydrolysis within intact heterolysosomes. 2. Particles from rats pre-treated with Trypan Blue, suramin or aurothiomalate released iodotyrosine more slowly than controls. Since these compounds are enzyme inhibitors that concentrate in liver lysosomes after administration *in vivo*, their effect is ascribed to intralysosomal inhibition of proteolysis. The doses used did not decrease endocytosis of albumin into liver or cause increased lysosome breakage during incubation, thus allowing some alternative explanations of the decreased proteolysis to be eliminated. Particulate carbon, a non-inhibitor that also concentrates in lysosomes, did not affect albumin hydrolysis.

It is becoming increasingly clear that the lysosome system is a peculiarly vulnerable target for drugs. This is because of the ready accessibility of lysosomes to exogenous materials by endocytosis and because of their ambivalent nature both as integral parts of the cellular machinery and as potential trouble-makers for cells. The various ways in which drugs may disturb lysosome function have been reviewed (de Duve, 1968; Allison, 1968). One such possible way is a decrease of the catabolic capacity of lysosomes after endocytosis of materials able to depress the activity of one or more of the lysosomal hydrolases. Such a mechanism has already been proposed to account for the action of the bisazo dye Trypan Blue in certain biological systems (Beck, Lloyd & Griffiths, 1967; Lloyd, Beck, Griffiths & Parry, 1968). Two other compounds that concentrate in lysosomes and are enzyme inhibitors are the trypanocide suramin (Smeesters & Jacques, 1968; Wills, 1952) and the anti-rheumatic sodium aurothiomalate (Persellin & Ziff, 1966; Norton, Lewis & Ziff, 1968; Ennis, Granda & Posner, 1968).

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The present experiments were undertaken to discover whether lysosomes laden with these three compounds could be shown to have a decreased digestive activity. The method used measures the rate of breakdown of endocytosed [^{125}I]iodinated albumin within isolated rat liver lysosomes and is based on that of Mego, Bertini & McQueen (1967). Some of the work has been reported briefly (Davies, Lloyd & Beck, 1969*a*,*b*).

MATERIALS AND METHODS

Chemicals. [^{125}I]Iodide was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. and bovine serum albumin (type II) and Triton X-100 from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Sodium aurothiomalate was obtained from May and Baker Ltd., Dagenham, Essex, U.K., and suramin was a gift from Imperial Chemical Industries Ltd., Macclesfield, Cheshire, U.K. A commercial sample of Trypan Blue (Williams Ltd., Hounslow, Middx., U.K.) was freed from salt and converted into the free acid form as described by Lloyd & Beck (1966). An aqueous 1% (w/v) solution of the dye was prepared for injections. All other chemicals were of analytical grade.

Preparation of denatured ^{125}I-labelled bovine serum albumin. ^{125}I-labelled bovine serum albumin was prepared by the chloramine-T procedure essentially as described by McConaghey & Dixon (1966). The labelled

protein, together with sufficient carrier bovine serum albumin to give a protein concentration of 1mg/ml, was denatured in 10% formaldehyde buffered to pH10 with 0.2M-Na_2CO_3–$NaHCO_3$ as described by Mego *et al.* (1967). In the final product less than 1% of the total radioactivity was soluble in trichloroacetic acid. This solution was stored at −15°C until required for injection.

Determination of protein. This was carried out by the method described by Lowry, Rosebrough, Farr & Randall (1951).

Animals. Male (250–300g) and female (180–200g) Wistar rats were used.

Preparation and incubation of lysosomes containing ^{125}I-labelled albumin. Non-starved rats were given intravenous injections into the femoral vein of denatured ^{125}I-labelled bovine serum albumin (2.5mg of protein/kg body wt.). Control animals had had no prior treatment, but other groups had received subcutaneous injections of Trypan Blue, suramin, aurothiomalate or carbon 24h before the injection of albumin. Rats were killed by a blow on the head 0.5h after the albumin injection, decapitated and bled. The liver was quickly removed and placed in ice-cold 0.25M-sucrose. To determine total liver radioactivity portions of liver were weighed and the radioactivity was counted in an automatic crystal-scintillation counter (Nuclear–Chicago). For experiments on isolated lysosomes liver (4g) was homogenized in ice-cold 0.25M-sucrose with a Potter–Elvehjem-type Teflon-on-glass homogenizer. The homogenate was diluted with 0.25M-sucrose to give a final volume of 35ml and centrifuged at 1000*g* for 10min at 4°C in a MSE High Speed 18 refrigerated centrifuge, with rotor 69181. The supernatant was decanted and centrifuged at 16500*g* for 20min and the resulting pellet was resuspended carefully in 20ml of 0.01M-tris–acetate buffer, pH7.4, containing 0.25M-sucrose and immediately incubated at 22°C. Samples were removed at intervals up to 2h and assayed for trichloroacetic acid-soluble radioactivity and for non-sedimentable trichloroacetic acid-insoluble radioactivity.

Measurement of trichloroacetic acid-soluble radioactivity. Two 0.5ml samples of the incubation mixture were removed at each time-interval, placed immediately into separate tubes containing cold 20% (w/v) trichloroacetic acid (0.5ml) and the total radioactivity was determined. The tubes were then centrifuged at 4°C to remove insoluble protein, the supernatants were decanted into fresh tubes and the radioactivity was re-counted to obtain the trichloroacetic acid-soluble radioactivity. The nature of the acid-soluble radioactivity was studied by gel filtration on Sephadex G-25 (Mougey & Mason, 1963).

Measurement of non-sedimentable trichloroacetic acid-insoluble radioactivity. Two further 0.5ml samples of the incubation mixture were removed at each time-interval, placed in ice-cold 3ml polyethylene tubes and centrifuged at 33000*g* for 20min. The resulting supernatants were placed into tubes containing 20% (w/v) trichloroacetic acid (0.5ml) and the total non-sedimentable radioactivity was counted. The tubes were then centrifuged to remove insoluble material and the supernatants decanted into fresh tubes. The non-sedimentable trichloroacetic acid-soluble radioactivity was then counted. This radioactivity was subtracted from the total non-sedimentable radioactivity to obtain the non-sedimentable trichloroacetic acid-insoluble radioactivity, a measure of the non-sedimentable labelled albumin.

RESULTS

Intralysosomal digestion of denatured ^{125}I-labelled albumin. When a suspension of rat liver lysosomes prepared from a rat injected with denatured ^{125}I-labelled albumin was incubated at 22°C in osmotically protected buffer, pH7.4, trichloroacetic acid-soluble radioactivity was released into the medium. In 37 separate experiments the percentage of total radioactivity that was soluble in trichloroacetic acid increased from 8.2 (S.D. ±2.5) at time 0 to 25.4 (±3.4) after incubation for 2h. Subtracting the initial from the final percentage for each individ-

Table 1. *Effects of intralysosomal Trypan Blue, suramin, aurothiomalate and carbon particles on the digestion of ^{125}I-labelled albumin within isolated rat liver heterolysosomes incubated at* 22°C

Experimental conditions were as described in the Materials and Methods section. The increase in acid-soluble radioactivity over 2h is used as a measure of digestion (see the Results section).

Compound	Dose (mg/kg body wt.)	No. of experiments	Increase in acid-soluble radioactivity (mean % of total radioactivity ± S.D.)
Control	—	37	17.2 ± 3.8
Trypan Blue	50	3	11.0 ± 1.3
	75	7	10.2 ± 2.4
	150	2	3.7*
Suramin	50	4	15.5 ± 2.3
	100	5	9.2 ± 2.1
	250	9	3.1 ± 1.0
Aurothiomalate	25	9	6.5 ± 2.3
	50	21	5.5 ± 4.4
	100	8	5.4 ± 2.4
Carbon particles	160	3	20.4 ± 2.9

* Mean of two determinations, 3.5 and 3.9

Table 2. *Effects of intralysosomal Trypan Blue, suramin, aurothiomalate and carbon particles on the stability of rat liver heterolysosomes laden with ^{125}I-labelled albumin*

Experimental conditions were as described in the Materials and Methods section. The increase in non-sedimentable acid-insoluble radioactivity over 2h is used as a measure of lysosome breakage or leakage (see the Results section).

Compound	Dose (mg/kg body wt.)	No. of experiments	Increase in non-sedimentable acid-insoluble radioactivity (mean % of total radioactivity ± S.D.)
Control	0	37	11.1 ± 4.5
Trypan Blue	50	3	12.5 ± 6.0
	75	7	12.2 ± 4.4
	150	2	11.4*
Suramin	50	4	10.5 ± 3.4
	100	5	13.4 ± 4.6
	250	9	13.0 ± 1.8
Aurothiomalate	25	9	11.9 ± 3.3
	50	21	15.9 ± 4.6
	100	8	19.6 ± 5.0
Carbon	160	3	13.0 ± 4.5

* Mean of two determinations, 13.7 and 9.0

Table 3. *Effects of procedures causing lysosome breakage on the release of trichloroacetic acid-soluble radioactivity and the initial concentration of non-sedimentable trichloroacetic acid-insoluble radioactivity during a 2h incubation of rat liver heterolysosomes laden with ^{125}I-labelled albumin*

Experimental conditions were as described in the Materials and Methods section.

Procedure	Increase in trichloroacetic acid-soluble radioactivity (% of total radioactivity)	Initial non-sedimentable trichloroacetic acid-insoluble radioactivity (% of total radioactivity)
Inclusion in of Triton X-100 (0.1%) incubation medium	1.0	80.4
Omission of sucrose from incubation medium	2.0	76.0
Freezing and thawing (10 cycles) before incubation	2.0	77.0

ual experiment yields a value of 17.2 (±3.8) for the average rise, i.e. the percentage of radioactivity becoming soluble in the incubation period. This value is used as a measure of the digestive capacity of control lysosomes and is included in Table 1. Sephadex G-25 chromatography of the acid-soluble radioactivity showed that the major component corresponded in elution pattern to iodotyrosine but not to iodide.

During the incubation at 22°C the non-sedimentable trichloroacetic acid-insoluble radioactivity increased from 2.3 (±1.0) to 13.4 (±5.2), showing that albumin originally bound within sedimentable structures becomes non-sedimentable without undergoing complete hydrolysis, this release being presumably due to lysosomal breakage. Subtracting initial from final percentage for each experiment yields a value of 11.1% (±4.5) for the average increase. This value is used as a measure of the degree of lysosome rupture in control experiments and is included in Table 2.

A number of experiments were carried out to establish that the digestion of denatured ^{125}I-labelled bovine serum albumin was taking place within membrane-bound structures. Table 3 shows the results of experiments in which the lysosomes were broken by the incorporation of 0.1% (w/v) Triton X-100, the omission of protective sucrose or ten cycles of freezing and thawing before incubation. In each case no significant increase in trichloroacetic acid-soluble radioactivity was observed and, as expected, the initial concentration of non-sedimentable trichloroacetic acid-insoluble radioactivity was high. In a further experiment (Table 4) it was shown that no digestion took place if granules (whether intact or broken)

Table 4. *Result of incubating liver lysosomes from untreated rats in tris buffer,* pH 7.4, *containing radioiodinated albumin*

Granules were prepared from uninjected rats and incubated as described in the Materials and Methods section. The incubation medium contained in addition 10 μg of radioiodinated albumin/ml. In some experiments Triton X-100 was added or the protective sucrose omitted.

Modification of incubation medium	No. of experiments	Increase in trichloroacetic acid-soluble radioactivity (%)	Recovery of added radioactivity in high-speed supernatant (%)
None (i.e. with 0.25 M-sucrose)	3	1.0	79.0
Presence of Triton X-100 (0.1%)	2	2.0	80.0
Omission of sucrose	2	1.0	76.0

Table 5. *Effects of Trypan Blue, suramin and aurothiomalate on the content of radioactivity in rat liver 0.5 h after injection of* ^{125}I*-labelled albumin*

Experimental conditions were as described in the Materials and Methods section. Three different batches of albumin were used for the three sets of experiments (with Trypan Blue, suramin and aurothiomalate respectively). Hence the last column, but not the penultimate, may be used for comparison of the effects of different compounds.

Compound	Dose (mg/kg body wt.)	No. of expts.	Radioactivity (c.p.m./g of liver)	% of control
Trypan Blue	0 (control)	3	102000 ± 2400	
	50	3	108400 ± 3100	106.3
	75	3	115200 ± 2600	112.9
	150	3	102300 ± 1900	100.3
Suramin	0 (control)	4	98000 ± 1840	
	25	4	112800 ± 6300	115.1
	100	4	98700 ± 2540	100.7
	250	4	82500 ± 7130	84.2
	500	4	43800 ± 300	44.7
	1000	4	14900 ± 460	15.2
Aurothiomalate	0 (control)	4	45000 ± 1830	
	25	4	53000 ± 5000	117.8
	50	4	50700 ± 4700	112.7
	100	4	52800 ± 2400	117.3

from an uninjected rat were incubated in buffer containing ^{125}I-labelled albumin.

Effects of Trypan Blue, suramin and aurothiomalate. Table 5 shows how pretreatment of rats with Trypan Blue, suramin or aurothiomalate affected the amount of total radioactivity in liver 0.5 h after injection of labelled protein. Except for the two highest doses of suramin, where much lower contents were found, there is no great departure from control values. This is taken to indicate that, with the same exception, the drugs studied did not greatly affect the extent of endocytosis of albumin in our experiments.

Tables 1 and 2 summarize the results obtained from incubations of rat liver lysosomes isolated from control rats and from animals pre-treated with various doses of Trypan Blue, suramin, aurothiomalate and carbon, the latter being chosen as an inert material known to concentrate in lysosomes (Meijer & Willighagen, 1961).

Trypan Blue. Trypan Blue lowered digestive capacity in a dose-dependent manner without affecting lysosome stability.

Suramin. The doses of suramin used (50, 100 and 250 mg/kg) were those which did not greatly affect the amount of radioactivity in the liver (Table 5). Within this dose range the extent of digestion was decreased in a dose-dependent manner, but there was no significant increase in the extent of lysosome breakage.

Aurothiomalate. Sodium aurothiomalate at 25 mg/kg caused a marked decrease in digestion but did not increase the extent of lysosome breakage over that in control suspensions. Increasing the dose to 50 or 100 mg/kg caused a slight further decrease in digestion accompanied by some increase of lysosome breakage.

Carbon. Carbon particles had no effect on either albumin digestion or lysosome stability.

Table 6 shows that the three inhibitors, if present

Table 6. *Effects of incubating liver lysosomes isolated from rats injected with radioiodinated albumin in media containing Trypan Blue, suramin or aurothiomalate*

Experimental conditions were as described in the Materials and Methods section, the incubation media containing in addition the compounds listed.

Addition		Increase in acid-soluble radioactivity (%)	Increase in non-sedimentable acid-insoluble radioactivity (%)
Trypan Blue	(1mg/ml)	20.0	11.4
Suramin	(1mg/ml)	15.9	7.9
	(10mg/ml)	18.8	12.3
Aurothiomalate	(1mg/ml)	16.8	8.4
	(10mg/ml)	16.2	12.4

in the incubation medium but not in the lysosomes, affect neither intralysosomal digestion nor lysosome stability.

DISCUSSION

Recent work has firmly established that one of the main functions of lysosomes is the intracellular digestion of macromolecules taken into the cell by endocytosis. Mego *et al.* (1967) described a method for monitoring digestion continuing within isolated mouse liver heterolysosomes. This was achieved by incubating liver particles from mice pre-treated with ^{131}I-labelled albumin in an osmotically protected medium. During a 2h incubation there was an increase in the percentage of total radioactivity in the suspension that was soluble in trichloroacetic acid. The nature of the acid-soluble radioactivity was studied and the major product identified as [^{131}I]iodotyrosine. Little or no iodide was detected. When the liver particles were suspended in non-protective medium or in the presence of Triton X-100, little increase in acid-soluble radioactivity was observed and the majority of the labelled protein could be recovered in a high-speed supernatant. From these and other experiments Mego *et al.* (1967) concluded that the iodotyrosine was derived from the labelled albumin by proteolysis within intact heterolysosomes. Studies on the degradation of radioiodinated albumin by cells in culture (Ehrenreich & Cohn, 1967; Unanue & Askonas, 1968; Gabathuler & Ryser, 1969) tend to support this view.

We have used the technique described by Mego *et al.* (1967) to study the effects of exogenous intralysosomal enzyme inhibitors on the digestive capacity of isolated lysosomes. Our techniques differ from theirs in a number of respects: we have used rats rather than mice, a more convenient isotope of iodine, and a lower incubation temperature. The latter change was made after preliminary experiments (Davies & Lloyd, 1968) showed extensive lysosome rupture during incubation at 37°C, particularly over the initial 30min. This finding is consistent with the results of Mego & McQueen (1967) on mouse liver granules incubated at 37°C.

The interpretation of the results shown in Tables 1 and 2 depends to a great extent on a number of preliminary experiments that show that release of labelled iodotyrosine from incubated granule suspensions proceeds only if the lysosomes remain intact. Thus the omission of sucrose from the incubation medium or the inclusion in the medium of Triton X-100 renders most of the labelled albumin non-sedimentable and effectively prevents digestion. A similar result follows if the granules are disrupted, before incubation, by freezing and thawing (Table 3). Moreover, labelled albumin is not digested if added to a suspension of rat liver granules suspended in either protective or non-protective medium (Table 4). It is thus established that breakdown of labelled albumin takes place only when the protein is within intact particles. This presumably reflects the considerable dilution of both enzymes and substrate, and the unfavourable pH conditions, which must follow upon lysosome breakage in this *in vitro* system.

Table 1 shows that the production of [^{125}I]-iodotyrosine is decreased in granules prepared from rats pre-treated with Trypan Blue, suramin or aurothiomalate. This decrease could result from a decreased availability of substrate (albumin) to the lysosomal enzymes. Such a situation might arise if a compound decreased endocytosis of albumin into liver cells. We therefore measured the effects of the three compounds on the total liver radioactivity 0.5h after injection (Table 5). This is, of course, not simply a measure of endocytosis, as the concentration at any time reflects the balance between uptake of albumin and its breakdown. However, except for the high (500 or 1000mg/kg) doses of suramin, which were not used for the studies on digestion (Tables 1 and 2), no significant effect on the total liver radioactivity was seen. Another explanation of decreased intralysosomal digestion relates to lysosomal stability. If granules from treated rats were more susceptible to rupture

during incubation, the amount of albumin available for proteolysis would be decreased; this follows because albumin, once released into the medium, is not further degraded (see above). To assess lysosomal stability, the extent of particle breakage has been estimated by the appearance of non-sedimentable albumin in the incubation (Table 2). This method depends for its validity on the observation that released albumin is not adsorbed to any great extent on particulate matter present in the suspensions (Table 3; Mego & McQueen, 1967). The results shown in Table 2 make it clear that altered lysosome stability cannot account for the decreased digestion observed in lysosomes from rats treated with the three drugs (Table 1). Nevertheless it is possible that the increase in lysosomal breakage that follows the two higher doses of aurothiomalate (Table 2) may be responsible for the slight differences in digestive capacity seen with different doses of this compound (Table 1).

In the light of these observations, the results given in Table 1 are most easily explained by the three compounds exerting their effects on the rate of protein digestion within digestive vacuoles (heterolysosomes). As stated in the introduction, each of these compounds localizes in liver lysosomes. Further there is evidence that Trypan Blue (Lloyd *et al.* 1968), suramin and aurothiomalate (M. Davies, unpublished work) inhibit acid proteinase of rat liver lysosomes as assayed with haemoglobin. In the case of suramin, the inhibition increased with the length of time the enzyme was in contact with the drug before incubation, suggesting that the inhibition is an irreversible denaturation. In contrast with the effects on digestion of Trypan Blue and suramin, the response to aurothiomalate is largely independent of dose within the range 25–100 mg/kg. Together with the observation (Ennis *et al.* 1968) that aurothiomalate inhibition of human synovial-fluid 'cathepsin' is reversed by cysteine, this raises the question of which of the lysosomal proteinases is affected by aurothiomalate. Cathepsin B, formerly thought to be a peptidase, has since been shown to have activity against polypeptides (Otto, 1970) and is dependent for activity on thiol groups. Cathepsin D, probably the principal proteinase of lysosomes, is not dependent on thiol groups and it would seem reasonable to speculate that the effect of aurothiomalate within lysosomes is likely to be on the activity of cathepsin B. The failure of aurothiomalate to suppress the iodotyrosine production beyond a certain value in our experiments would be explained if a proportion of the iodotyrosine were released from albumin without the intervention of cathepsin B.

The experiments described in this work demonstrate that isolated liver lysosomes from rats pretreated with the enzyme inhibitors Trypan Blue, suramin and aurothiomalate have a decreased digestive capacity. This phenomenon is not seen in liver granules prepared from rats treated with carbon particles nor if the inhibitors are in the incubation medium but not in the granules. For these reasons and others developed above, it is probable that the decreased digestion is caused by intralysosomal inhibition of proteolytic enzymes. However certain other possible mechanisms, such as impairment of the fusion between lysosomes and phagosomes, cannot be excluded at this time.

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