J. Hyg., Camb. (1974), **72**, 297
Printed in Great Britain

Interactions of TRIC agents with macrophages: effects on lysosomal enzymes of the cell

By JANICE TAVERNE,* W. A. BLYTH,† and R. C. BALLARD‡

Medical Research Council Trachoma Unit,
The Lister Institute of Preventive Medicine,
Chelsea Bridge Road, London SW1W 8RH

(*Received* 16 *August* 1973)

SUMMARY

Two changes were observed in the acid phosphatase of macrophages that had ingested infective TRIC organisms: the proportion of extralysosomal enzyme rose, while the total amount in the cells fell. Both effects were directly related to the number of organisms ingested and increased with time. When macrophages were inoculated with more than 50 organisms per cell, changes were obvious within a few hours; with 2–10 organisms per cell changes were detectable only after 18 hr. or more. Enzyme appeared in the culture medium as the amount in the cells decreased. Ingestion of organisms killed by heat or treated with antibody did not induce such changes. In infected BHK-21 cells, no changes in acid phosphatase were detected at any stage of the developmental cycle of the organism.

INTRODUCTION

In people suffering from trachoma, damage to the epithelial cells of the conjunctiva is often severe and it may result, at least in part, from pathological changes in macrophages in the exudate (Taverne & Blyth, 1971). Furthermore, intravenous injection of chlamydiae into mice causes toxic and sometimes lethal reactions that are associated with rapid injury to the reticulo-endothelial system, in particular to macrophages (Schoenholtz, 1962).

Macrophages inoculated *in vitro* with the agents of trachoma or inclusion conjunctivitis (TRIC agents) undergo a cytotoxic reaction and die. Vacuolation was observed in the cytoplasm of the cells before changes in the permeability of the cell membrane were detected (Taverne & Blyth, 1971). Similar effects caused by bacterial endotoxins have been related to their action on lysosomes, with the subsequent release of lysosomal enzymes (Weissmann & Thomas, 1962). Thus it seemed likely that the toxic activity of TRIC agents might also be mediated through the cell's lysosomes, particularly in view of the contrast observed by electron microscopy between events occurring in macrophages and those in

* Present address: Department of Pathology, Royal College of Surgeons, Lincolns Inn Fields, London WC2A 3PN.

† Present address: Department of Bacteriology, The University of Bristol, University Walk, Bristol.

‡ Present address: Poliomyelitis Research Foundation, South African Institute for Medical Research, Johannesburg.

BHK-21 cells that have ingested TRIC agents (Lawn, Blyth & Taverne, 1973); in macrophages the organisms entered lysosomes; but in BHK-21 cells, in which they multiply without inducing cytotoxic effects, the organisms developed outside the lysosomal system.

We therefore tested the hypothesis that the ingestion of TRIC organisms by macrophages is followed by alterations in the activity of lysosomal enzymes in the cells; and using acid phosphatase as an indicator, we investigated the possibility that such changes might be used as the basis of a quantitative test for the toxicity of TRIC organisms for macrophages.

METHODS

Methods for the culture of BHK-21 cells and macrophages, and for the preparation of suspensions of TRIC agents grown in yolk sacs and their titration in BHK-21 cells, have been described in detail (Taverne & Blyth, 1971).

TRIC agents

The fast-killing variant (Reeve & Taverne, 1963) of strain TRIC/2/SAU/HAR-2/OT (Murray *et al.* 1960) was used as suspensions made either from infected yolk sacs or from infected BHK-21 cells. The latter were prepared by disrupting cells 42–46 hr. after infection by shaking them with glass beads or by sonication in an ultrasonic cleaning bath. In experiments with cockerel antiserum, the fast-killing variant of MRC-4 (Jones, 1961) was used.

Macrophage cultures

Peritoneal exudate cells from CS1 mice which had not received peritoneal irritants were seeded into flat-bottomed plastic tubes (Conway tubes, Turner-Stayne Laboratories, Bishop Auckland, Co. Durham). Glass coverslips were included only when examination under the microscope was required. Cultures were used after overnight incubation at 35° C. in Eagle's MEM medium (Wellcome Reagents Ltd.) containing 40 % horse serum in an atmosphere of 5 % CO_2 in air. They usually then contained $2–5 \times 10^5$ cells per tube.

Inoculation of cultures

The culture medium was replaced with 1 ml. of Eagle's MEM medium supplemented with 10 % horse serum (MM) containing the required number of TRIC organisms; the tubes were centrifuged at 600 ***g*** for 30 min. at 37° C. The medium was then replaced with fresh MM.

Antiserum against TRIC agents

A cockerel was given six intraperitoneal injections at weekly intervals of a suspension of MRC-4*f*, each equivalent to one infected yolk sac. It was bled 8 days after the last injection.

Measurement of acid phosphatase activity in cell cultures

To determine the total amount of acid phosphatase (ACPase) activity in the cells, the medium was replaced with 2 ml. of 0·05 M citrate buffer pH 5·0 containing 0·003 M *p*-nitrophenol phosphate (Sigma Corporation) and 0·05 % Triton X100 (Sigma Corporation). To determine the amount of extralysosomal ACPase, the medium in replicate cultures was replaced with 2 ml. of substrate in buffer without Triton X100. The tubes were then incubated at 37° C. for 30 min. after which the enzyme reaction was stopped by adding 2 ml. N-NaOH. This also lysed the cells so that the *p*-nitrophenol formed by the action of the enzyme was released into the supernatant fluid; its optical density (OD) was measured at 405 nm. All tests were done on groups of three replicate cultures and activities were expressed in terms of mean OD405. In some experiments, the culture medium was transferred to clean tubes and assays of the total and extralysosomal ACPase activity were done as above.

Measurements of ACPase activity in yolk-sac suspensions

Duplicate volumes of suspension were incubated with *p*-nitrophenol phosphate in citrate buffer containing Triton X100 and the reaction was stopped with NaOH as above; OD405 readings were made against a blank containing an equivalent amount of yolk-sac material, prepared by mixing 2 ml. substrate in buffer containing Triton X100 with 2 ml. NaOH and then adding 1 ml. of the yolk-sac suspension.

β-Glucuronidase assays

Since mouse macrophages contain 10 times less β-glucuronidase than ACPase, overnight cultures in small plastic flasks (Falcon Plastics Ltd.) were used. The medium from duplicate flasks, each containing 3×10^6 macrophages, was replaced with 2 ml. of 0·001 M *p*-nitrophenol glucuronide (Sigma Corporation) in 0·05 M citrate buffer pH 5·0 or 2 ml. of substrate in buffer containing 0·05 % Triton X100. The flasks were incubated at 37° C. for 1 hr., the reaction was stopped with 2 ml. of 0·1 M glycine in 0·1 M-NaCl at pH 10·0 and OD405 was determined.

RESULTS

Previously we demonstrated that macrophages inoculated with TRIC organisms underwent a cytopathic effect, the severity of which depended both on the number of infective organisms ingested by each cell and the time after inoculation at which the cultures were examined (Taverne & Blyth, 1971). To relate these observations to lysosomal changes, groups of cultures were inoculated with various numbers of organisms per cell and intracellular ACPase activity was measured immediately before and at intervals after centrifugation. In control cultures, that had received either various concentrations of a normal yolk-sac (NYS) suspension or fresh culture medium, the total amount of ACPase per 10^6 cells gave a mean OD405 of 2·99 (S.E. ±0·14); it did not alter significantly during

Table 1. *Changes in ACPase activity in macrophages inoculated with TRIC organisms*

	Control cultures		Inoculated cultures		
Hours after end of centrifugation	Total* ACPase	Extra-lysosomal ACPase†	Infective organisms per cell	Total* ACPase	Extralysosomal ACPase†
Before centrifugation	0·933	0·067		—	—
0	1·037	0·098		0·887	0·205
1½	—	—		0·707	0·356
3	1·006	0·095	200	0·564	0·292
4½	—	—		0·511	0·218
6	1·111	0·106		0·415	0·194
0	1·050	0·065		0·893	0·069
3	—	—	50	0·860	0·069
5	—	—		0·756	0·219
0	0·620	0·117		0·632	0·138
6	—	—		0·530	0·088
12	0·558	0·113	10	0·394	0·108
18	—	—		0·346	0·088
24	0·781	0·093		0·412	0·076

* Mean OD 405: assayed in presence of Triton X100.

† Mean OD 405: assayed in absence of Triton X100.

In the experiment with 200 organisms per cell, control cultures received an equivalent concentration of normal yolk-sac suspension; otherwise the organisms used were grown in BHK-21 cells and control cultures were incubated with fresh medium.

experiments lasting 6 hr., although over longer periods it sometimes showed a slight increase. In these control cultures the average proportion of enzyme outside the lysosomes was 10·5 % (S.E. ±0·74) of the total; a rise to 25 % or more was taken as a sign that the cultures were unhealthy and results from experiments in which they were used were ignored.

Similar measurements with infected cultures revealed that the total amount of ACPase in the cells diminished during the 24 hr. period of observation after infection; the more organisms per cell, the sooner the diminution was observed. The results of representative experiments with three different multiplicities of infection are given (Table 1).

When the total amount of enzyme present in inoculated cells was expressed as a percentage of that in control cultures at the time of inoculation (or, in longer experiments, in control cells at the time of sampling) the loss of enzyme observed with time was clearly demonstrated. The rate of loss depended on the multiplicity of infection, as shown for the first 6 hr. after incubation with 50–500 infective organisms per cell (Fig. 1) and during 24 hr. with 2 or 10 organisms per cell (Fig. 2). Accompanying the decreases in the total amount of ACPase, changes in the amount of extralysosomal enzyme were also observed, in that during the first 2–6 hr. after infection there was sometimes more free enzyme present than in control cells (Table 1). These effects were induced by four strains of TRIC agent tested, whether the organisms were cultured in BHK-21 cells or in chick

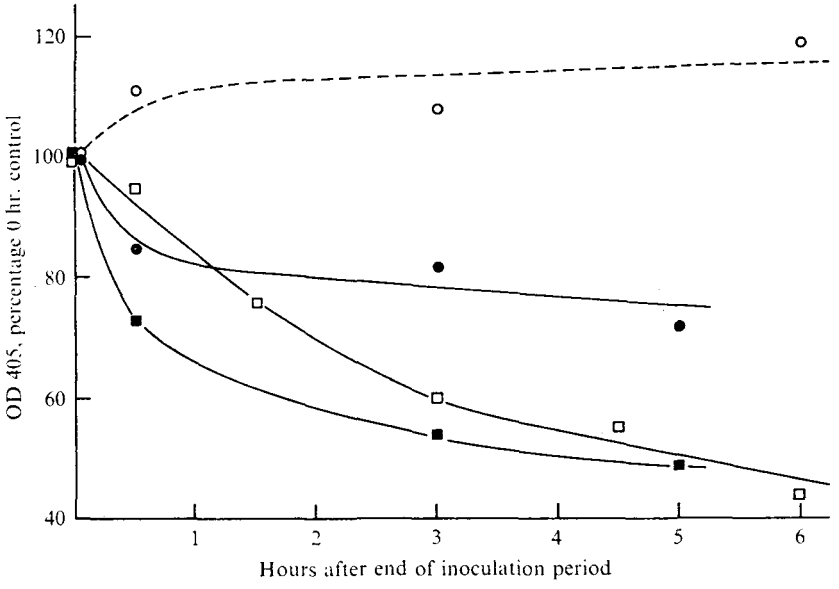

Fig. 1. Decrease of ACPase activity with time, in macrophages inoculated with large numbers of TRIC organisms. ○, Normal yolk sac; ●, 50 organisms per cell; □, 200 organisms per cell; ■, 500 organisms per cell.

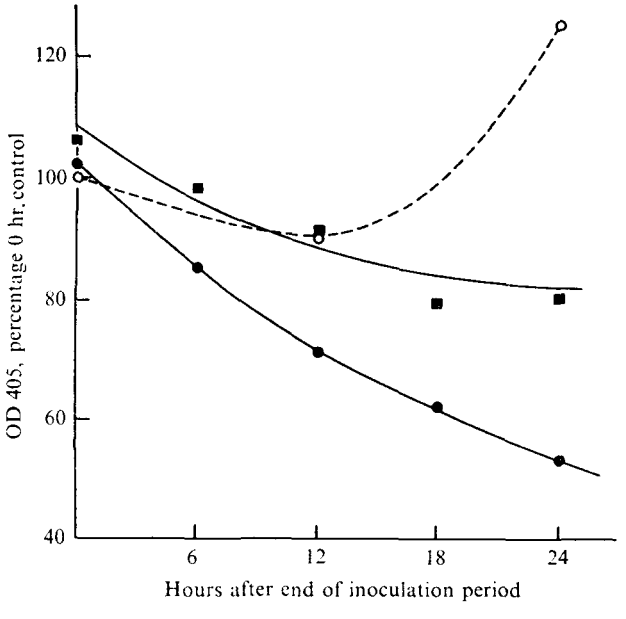

Fig. 2. Decrease of ACPase activity with time, in macrophages inoculated with small numbers of TRIC organisms. ○, Medium only; ■, two organisms per cell; ●, 10 organisms per cell.

https://doi.org/10.1017/S0022172400023512 Published online by Cambridge University Press

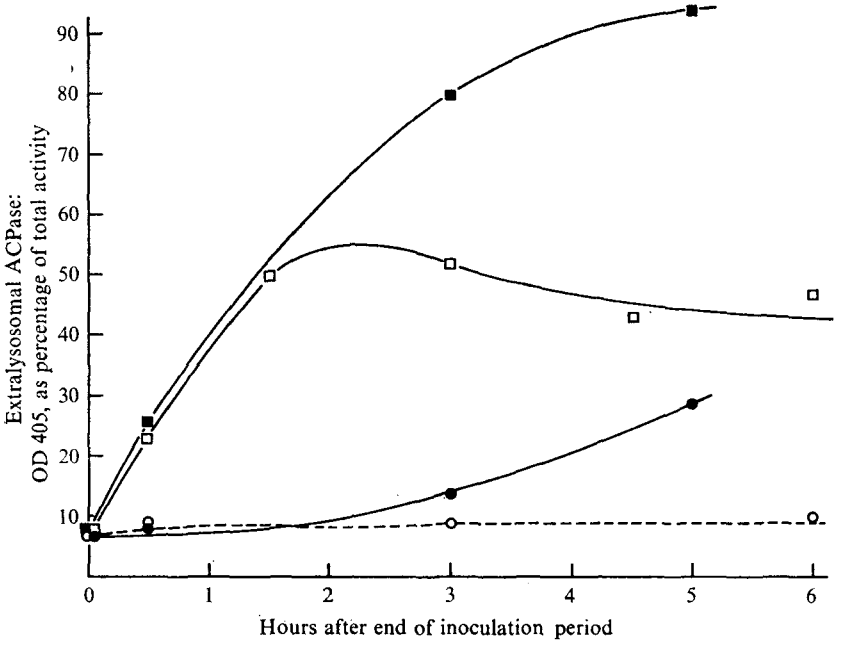

Fig. 3. Increase in extralysosomal ACPase activity with time, in macrophages inoculated with large numbers of TRIC organisms. ○, Normal yolk sac; ●, 50 organisms per cell; □, 200 organisms per cell; ■, 500 organisms per cell.

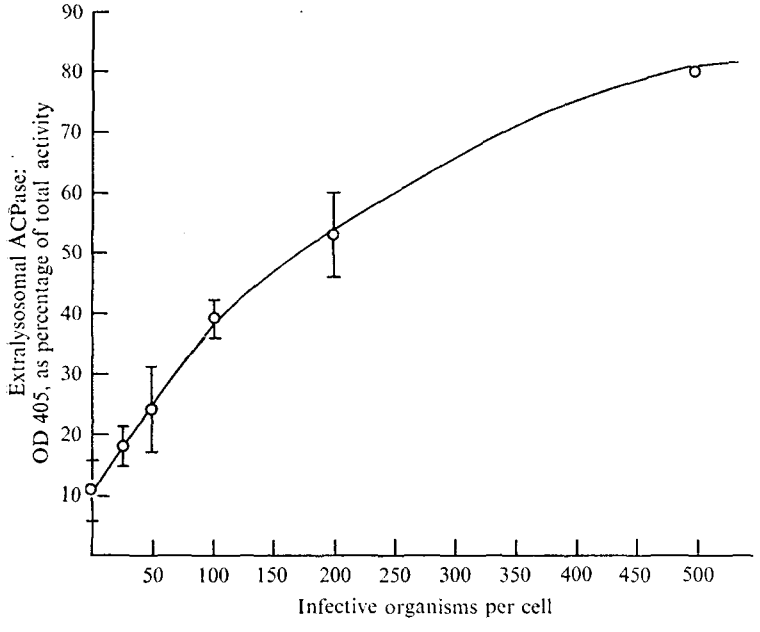

Fig. 4. Increase in extralysosomal ACPase activity with number of TRIC organisms in macrophages 3 hr. after inoculation. ϕ, Mean rate with standard deviations.

https://doi.org/10.1017/S0022172400023512 Published online by Cambridge University Press

embryo yolk sacs. The changes in the amount of extralysosomal enzyme are more obvious when expressed as a percentage of the total activity present in the cells at each time (Fig. 3). With 500 organisms per cell the concentration of free enzyme rose steadily until virtually all the enzyme remaining in the cells was free 5 hr. after centrifugation; with 200 organisms the proportion free reached a maximum of about 50 %, 3 hr. after centrifugation. In cultures inoculated with less than about 50 organisms per cell the proportion free was not significantly different from that in the controls during the first 5 hr. after inoculation, and rarely rose above 30 % of the total during the next 18 hr. Thus at any time from 1 to 3 hr. after inoculation there was a direct relationship between the amount of ACPase released from the lysosomes (but remaining within the cells) and the number of organisms inoculated per cell; this is illustrated for the 3 hr. sample (Fig. 4).

ACPase activity in culture medium

Since it seemed likely that the decrease in the total amount of ACPase in macrophages that had ingested viable TRIC organisms might be due to leakage of the enzyme from the cytoplasm into the extracellular fluid, ACPase activity in the culture medium was measured at various times after the inoculation of different numbers of organisms. Yet again, the enzyme activity showed a relationship between dose and time after inoculation. For instance, with 64 organisms per cell, about 20 % of the total activity in the culture was found in the medium 2 hr. later; after 20 hr. this increased to 40 %, whereas with two organisms per cell, only 16 % was found in the medium 24 hr. after inoculation.

ACPase activity in the culture medium measured in the presence of Triton X 100 was frequently lower than in its absence; indeed on some occasions there was apparently no activity although it was still measurable in the absence of the detergent. When medium from cultures inoculated 2 hr. previously with 200 infective organisms per cell was centrifuged at 8000 ***g*** for 10 min. – conditions in which lysosomes are deposited – about 75 % of the ACPase activity remained in the supernatant fluid.

β-Glucuronidase activity in macrophages

To verify that results obtained with ACPase were representative of other lysosomal enzymes, macrophage cultures were inoculated with 10 TRIC organisms per cell and both β-glucuronidase and ACPase activities were assayed in the presence of Triton X 100 24 hr. later.

As with ACPase, the amount of β-glucuronidase diminished with time, although to a lesser extent: 24 hr. after infection the cells contained 59 % of the β-glucuronidase activity and 27 % of the ACPase activity present at the end of the inoculation period.

Effect on ACPase activity of organisms killed by heat

Heat-killed TRIC organisms are not toxic to mice when injected intravenously, and except in massive doses, are not cytotoxic for macrophages in culture

https://doi.org/10.1017/S0022172400023512 Published online by Cambridge University Press

Table 2. *Protection by specific antiserum against the action of TRIC agents on the acid phosphatase of macrophages*

No. of organisms per cell	Serum	Total ACPase: % of that in uninoculated cells	% extra-lysosomal ACPase
None	Normal	100	5
	Antiserum	83	8
100	Normal	38	36
	Antiserum	82	12
1000	Normal	36	60
	Antiserum	86	21

Organisms were incubated with a 1/4 dilution of serum at 37° C. for 30 min. before inoculation; assays were done 4 hr. later.

(Taverne & Blyth, 1971). Various concentrations of organisms – up to 200 per cell – that had been killed by incubation overnight at 56° C. were centrifuged onto macrophage cultures and ACPase activity was measured at intervals up to 6 hr. after centrifugation. With a dose of 80 or more organisms per cell there was an insignificant decrease in the amount of enzyme present (to a mean of 90 % of control values) and some release of free enzyme (mean 21 % of total), but there was no increasing effect with time.

Protection by specific antiserum

To establish that changes observed in the ACPase of macrophages were caused specifically by the TRIC organisms in the inoculum, suspensions containing 1×10^7 or 1×10^8 infective organisms were incubated with an equal volume of fowl antiserum diluted 1/4 at 37° C. for 30 min. The mixtures were then used to inoculate macrophage cultures at a concentration (based on the original infectivity of the suspension) of 1000 or 100 organisms per cell; ACPase was assayed 4 hr. later (Table 2). In macrophages of control cultures that received 100 organisms per cell the amount of ACPase activity fell to 38 % of that in uninoculated cultures, and 36 % of the activity present was extralysosomal; but in those that received the same dose of organisms incubated with antiserum both the total amount and the proportion free were the same as in uninoculated cells. Even in cultures inoculated with 1000 organisms per cell no significant changes were caused by organisms treated with antibody. The antiserum had a neutralization titre of 1/12 in terms of a 50 % reduction of inclusion count in a neutralization test in BHK-21 cells (Blyth & Taverne, 1974); a 1/3 dilution protected 50 % of mice against the toxic effect of 4 LD50 injected intravenously. Similar results were obtained with an antiserum prepared in mice.

Preliminary experiments were done on the behaviour of macrophages from mice immunized with TRIC organisms by intravenous injection 10 days earlier. Inoculation of these macrophages with infective organisms induced greater changes in both the total amount of ACPase and in the proportion that was

Table 3. *The relative amounts of ACPase activity and infective organisms deposited by centrifugation through various concentrations of sucrose*

Sample	Molarity of sucrose	Centrifugation ×*g*	Centrifugation min.	ACPase* activity	Infectivity†	OD 405/ 10^6 IFU
Unpurified yolk sac	None	—	—	585	$2{\cdot}6 \times 10^8$	2·25
	None	8,000	30	156	$3{\cdot}1 \times 10^8$	0·5‡
Suspension of sample marked‡ in 0·25 M sucrose	0·25 M	8,000	20	45	$1{\cdot}2 \times 10^8$	0·36
	0·5	8,000	20	15	$4{\cdot}5 \times 10^7$	0·33
	1·0	8,000	20	9	$3{\cdot}9 \times 10^7$	0·23
	0·25	10,000	60	69	$5{\cdot}1 \times 10^8$	0·14
	0·5	10,000	60	24	$5{\cdot}4 \times 10^8$	0·044
	1·0	10,000	60	3	$2{\cdot}4 \times 10^8$	0·013

* OD 405/ml. adjusted to volume of original sample.
† Inclusion forming units (IFU)/ml. adjusted similarly.

extralysosomal than in cells from unimmunized mice. It thus appeared that these cells were sensitized to the action of the organisms, but, paradoxically, the protection afforded by antiserum was greater with cells from immunized than from normal mice.

ACPase activity in yolk-sac suspensions

In later experiments on changes in the ACPase of macrophages after the ingestion of TRIC organisms the effects reported above were not always reproduced. Inoculation with some infected yolk-sac suspensions was followed by an increase in the total amount of ACPase in the cells, instead of a decrease. Sometimes the amount rose as high as 150 % of the control immediately after centrifugation, and this value was maintained for at least 5 hr. There was also an increase above that expected in the extralysosomal enzyme. Further investigation revealed that the suspensions giving anomalous results themselves contained significant quantities of ACPase.

By chance, the suspensions used earlier had possessed very high infectivity titres ($> 5 \times 10^9$ infective organisms/ml.) so that although they too contained ACPase, it had apparently been diluted out enough not to interfere with measurements of the enzyme activity in macrophages, even when the multiplicity of infection was high. Suspensions of organisms made from infected BHK-21 cells usually contained relatively less ACPase activity than yolk-sac suspensions, and as they also possessed high infectivity titres results obtained with them generally agreed with those of the early experiments. No simple relationship could be found, however, between the amount of activity in a suspension and increase in activity in the macrophage cultures after inoculation with that suspension. Attempts were therefore made to separate the enzyme activity of the suspension from the organisms.

Since the last stage of purification of yolk-sac suspensions was centrifugation at 8000 *g* for 15 min., the enzyme activity must have been sedimented with the organisms. It must therefore still have been attached to lysosomal membranes, although the lysosomes themselves cannot have been intact since more than 90 %

Table 4. *The effect of purified and unpurified TRIC organisms on the ACPase of macrophages inoculated with* 200 *organisms per cell*

Yolk-sac suspension	ACPase activity in inoculum*	Total ACPase in cells†		Extralysosomal ACPase in cells‡	
		0 hr.	4 hr.	0 hr.	4 hr.
Before treatment	136	144	124	56	46
After purification§	5	104	85	27	23

* OD 405 units per ml.

† Total activity in cells measured in presence of Triton X100 expressed as percentage of uninoculated controls.

‡ Activity in absence of Triton X100 expressed as percentage of total activity in those cultures.

§ Centrifugation through 1·0 M sucrose at 10,000 *g* for 60 min.

of the activity was detected by incubation with *p*-nitrophenol phosphate in the absence of Triton X 100. The particles bearing ACPase were, however, separable from infective TRIC organisms by centrifugation through dense solutions of sucrose. To determine the best conditions for separation, an unpurified 20% suspension of infected yolk sac was centrifuged at 8000 *g* for 30 min. and the pellet was resuspended to the original volume in 0·25 M sucrose in phosphate buffered saline pH 7·4. One ml. volumes of this suspension were layered onto 40 ml. volumes of buffer each containing a different concentration of sucrose and centrifuged at 4° C., either at 8000 *g* for 20 min. or 10,000 *g* for 60 min. The deposits were resuspended in 0·25 M sucrose in buffer and the ACPase activity was assayed and related to the number of infective organisms determined by titration in BHK-21 cells (Table 3).

Although centrifugation of crude yolk-sac material in the absence of sucrose removed much ACPase activity, the higher the concentration of sucrose the greater the separation of ACPase and infectivity achieved. Centrifugation at 8000 *g* through 1·0 M sucrose did not deposit all the organisms, but all were deposited with the least ACPase contamination per infective organism when centrifuged through 1·0 M sucrose at 10,000 *g* for 60 min.

To verify that when ingested by macrophages TRIC organisms purified in this way behaved like organisms from infected cell cultures – or in yolk-sac suspensions of high titre – and did not induce an increase in the total activity of ACPase, macrophage cultures were inoculated with a suspension that had been centrifuged through 1·0 M sucrose for 60 min.; duplicate cultures received the same suspension, untreated, as a control. The multiplicity of infection for the untreated suspension was calculated to be 200 infective organisms per cell and the purified suspension was used at an equivalent dilution. ACPase in the cells was assayed at the end of the inoculation period and 4 hr. later (Table 4). As before, the untreated suspension caused an increase both in total and extra-lysosomal enzyme activity. By contrast, the purified material caused no significant increase in total activity at 0 hr.: the results obtained at 4 hr., however, suggest that the multiplicity of infection of the purified suspension was lower

than the estimated 200 organisms per cell (Unfortunately, the infectivity titrations on this inoculum failed because the BHK-21 cells were unhealthy). Nevertheless, our findings indicate that centrifugation through sucrose provides a useful method for removing ACPase activity from suspensions to be used in experiments of this kind. It should be noted, however, that in preliminary experiments suspensions purified in this way tended to lose infectivity if stored at $-70°$ C. in 0·25 M sucrose in buffer.

Effect of TRIC organisms on ACPase of BHK-21 cells

By contrast with the toxic effect of TRIC organisms on macrophages, no cytopathic effects are seen during the growth cycle of the organism in BHK-21 cells, apart from the developing inclusion. To discover if lysosomal reactions were also different, cultures were infected with 1, 10, 50 or 100 organisms per cell and the total amounts of ACPase and extralysosomal portion were monitored at intervals during the growth cycle. No significant changes in enzyme activity were observed. Even an inoculum of 450 infective organisms per cell caused no changes in ACPase activity 2 hr. after infection, although by this time 60 % of the activity was extralysosomal in macrophages inoculated with the same dose of organisms.

DISCUSSION

In earlier work on the effect of ingested particles on the lysosomes of macrophages, the cells were usually disrupted mechanically, the suspension was centrifuged to deposit lysosomes and enzyme activity was assayed in both deposit and supernatant fluid. In this way, Cohn & Wiener (1963) detected a difference in the distribution of ACPase between the lysosomal and non-lysosomal fractions obtained 2 hr. after macrophages had ingested as few as 15 heat-killed *Escherichia coli*. These workers concluded, with some caution, that although the enzyme was apparently released from the lysosomes, it was likely that it was merely redistributed into secondary lysosomes, and that as these were bigger and perhaps more fragile they broke up during the process of cell disruption. In experiments not reported here we ensured that our method of assay of ACPase was as sensitive as those that involved mechanical disruption of cells and also that during incubation with enzyme substrate at pH 5·0 the cells remained intact. Using our system of assay we therefore repeated the experiment of Cohn & Wiener with macrophages that had ingested 15 heat-killed *E. coli*; the proportion of extralysosomal ACPase was no different from that in control cultures. We conclude that the ingestion of such dead bacteria does not induce the release of lysosomal enzymes into the cytoplasm of the macrophage, and that the presence even of dead bacteria in the lysosomes probably makes them more susceptible to mechanical disruption.

By contrast, ingestion of infective TRIC organisms by macrophages leads to profound changes in the cell's lysosomal enzymes. Measurements on one such enzyme, acid phosphatase, indicate that the extralysosomal activity increases *pari passu* with a decrease in the total amount of activity in the cells; analogous

results were obtained with another lysosomal enzyme, β-glucuronidase. That these changes are caused by the organisms themselves is shown by the protective effect of specific antiserum against TRIC agents; the dependence of the changes upon infectivity is shown by the lack of reactivity of heat-killed organisms. The alterations in hydrolase activity closely parallel the morphological damage reported previously. For both phenomena, the time of onset and the severity of the observed changes depend on the number of organisms ingested. Both also show the same degree of sensitivity; 50 or more organisms per cell cause dramatic morphological changes within a few hours, but when a few organisms are ingested by each cell these changes are not manifest until the next day.

By contrast with the lysosomal reactions that occur in macrophages that have ingested TRIC organisms, no leakage was observed from the lysosomes of BHK-21 cells at any stage during the multiplication cycle of the organism, even with heavy multiple infection. This contrast in behaviour between the two types of cell can be explained by the observation that, whereas in macrophages TRIC organisms enter secondary lysosomes and are degraded, in BHK-21 cells they multiply in inclusions that never fuse with lysosomes (Lawn *et al.* 1973).

Our findings are best explained by the hypothesis that, in macrophages, infective TRIC organisms interact with and damage the lysosomal membrane; the consequent release of the contents in soluble form, first into the cytoplasm of the cell and then into the culture medium causes the morphological damage. The absence of cytotoxicity of TRIC agents for the cell in which they multiply contrasts with the effects of the agent of psittacosis multiplying in L cells (Kordova, Wilt & Sadiq, 1971). Cytopathic effects were reported which were accompanied by the release of lysosomal enzymes towards the end of the growth cycle. It is likely that the cell lysis was related to the development of the new generation of organisms; there is no evidence that lysosomes were directly involved. That TRIC agents do not induce these effects may be related to their tendency to remain within a circumscribed inclusion which can remain intact even after extrusion from the cell (Blyth, Taverne & Garrett, 1971). In virus infections too, effects on lysosomes, cell lysis and the manner of release may be related. Wolff & Bubel (1964) found that with vaccinia – which leaks from cells slowly without lysis – there was no alteration of lysosomal enzyme activity; whereas with poliovirus there was rapid release of both virus and lysosomal enzymes, associated with lysis of the host cell.

The simplicity and reproducibility of our technique makes it useful as a routine method for measuring the toxicity of suspensions of TRIC agents, in preference to tests based on the death of mice and calculation of LD 50. If large numbers of organisms are available ($>$ 50 per cell), assay of extralysosomal activity provides a measure of toxicity within a few hours. This rapid test is no more sensitive than assay by intravenous injection in 10 g. mice since the inoculum required for the macrophage culture – about 10^7 live organisms – is equivalent to 1 LD 50. Suspensions containing fewer organisms can, however, be assayed *in vitro* if measurements are made of the total amount of activity in macrophages about 24 hr. after inoculation.

We wish to thank Mrs Anne Fitzpatrick for her excellent technical help, Dr G. P. Manire for providing us with antiserum and Dr A. M. Lawn for many stimulating discussions.

REFERENCES

Blyth, W. A., Taverne, J. & Garrett, A. J. (1971). Trachoma inclusions separated from cells. In *Trachoma and Related Disorders. Proceedings of a Symposium Held in Boston, Mass. August* 1970 (ed. R. L. Nichols), pp. 79–87. Amsterdam: Excerpta Medica.

Blyth, W. A. & Taverne, J. (1974). Neutralization of TRIC organisms by antibody: enhancement by antisera prepared against immunoglobulins. *Journal of Hygiene* **72**, 129.

Cohn, Z. A. & Wiener, E. (1963). The particulate hydrolases of macrophages. II. Biochemical and morphological response to particle ingestion. *Journal of Experimental Medicine* **118**, 1009–20.

Jones, B. R. (1961). Trachoma and allied infections. *Transactions of the Ophthalmological Society of the United Kingdom* **81**, 367–78.

Kordova, N., Wilt, J. C. & Sadiq, M. (1971). Lysosomes in L cells infected with *Chlamydia psittaci* 6BC strain. *Canadian Journal of Microbiology* **17**, 955–9.

Lawn, A. M., Blyth, W. A. & Taverne, J. (1973). Interactions of TRIC organisms with macrophages and BHK-21 cells observed by electron microscopy. *Journal of Hygiene* **71**, 1–14.

Murray, E. S., Bell, S. D. Jr., Hanna, A. T., Nichols, R. L. & Snyder, J. C. (1960). Studies on Trachoma. 1. Isolation and identification of strains of elementary bodies from Saudi Arabia and Egypt. *American Journal of Tropical Medicine* **9**, 116–24.

Reeve, P. & Taverne, J. (1963). Observations on the growth of trachoma and inclusion blennorrhoea viruses in embryonate eggs. *Journal of Hygiene* **61**, 67–75.

Schoenholtz, W. K. (1962). Experimental Studies on Lethal and Immune Mechanisms in Massive *Bedsonia* Infections. PhD Dissertation, University of California, Berkeley, California.

Taverne, J. & Blyth, W. A. (1971). Interactions between trachoma organisms and macrophages. In *Trachoma and Related Disorders. Proceedings of a Symposium Held in Boston, Mass. August* 1970 (ed. R. L. Nichols), pp. 88–107. Amsterdam: Excerpta Medica.

Weissmann, G. & Thomas, L. (1962). Studies on lysosomes. 1. The effects of endotoxin, endotoxin tolerance and cortisone on the release of acid hydrolases from a granular fraction of rabbit liver. *Journal of Experimental Medicine* **116**, 433–50.

Wolff, D. A. & Bubel, H. C. (1964). The disposition of lysosomal enzymes as related to specific viral cytopathic effects. *Virology* **24**, 502–5.