THE SUBCELLULAR DISTRIBUTION OF ANTIGEN IN MACROPHAGES

By E. KÖLSCH,* Dr. rer. nat., and N. A. MITCHISON, D. Phil.

(From the National Institute for Medical Research, London, N.W. 7, England)

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Increasing evidence indicates that initiation of an immune response often requires the cooperation of functionally different cell types. It has been suggested that the decision whether protein antigens induce an immune response or paralysis depends on whether or not they are taken up by macrophages (for review see references 1, 2).

Several antigens have been found more potent in immunization if administered in a macrophage-bound instead of a free-form (3–6). This effect is strongest for those antigens which normally require large doses for immunization; when the effect is weak, or even reversed (7), this can be attributed to efficient phagocytosis after administration of the free form of the antigen (5, 8). Efficient phagocytosis is not, however, the sole prerequisite for immunogenicity. Pneumococcal polysaccharides and copolymers of D-amino acids are efficiently taken up and retained by macrophages (9–11), but nevertheless tend to induce paralysis (9, 12).

Studies on the fate of antigen after uptake by macrophages have revealed that a small amount of material is retained in an immunogenic form over long periods, although the major part is degraded quickly and the degradation products released (13–15). Antigen has been found within organelles which have been identified by electron microscopy as phagolysosomes (16). Lysosomal particles from lymphatic tissue appear to be functionally heterogeneous (17), and so the site of long-term retention may be distinct from those phagolysosomes and lysosomes in which the bulk of antigen is found shortly after uptake (18–20).

Theories of macrophage function vary widely from those which assign to them a role of nonspecific concentration, retention, and presentation of antigen (5, 6, 21), to those of transfer of informational RNA (22). The present study of the intracellular fate of antigen has the aim of elucidating this role. A search is included for subcellular deficiencies in macrophages from X-irradiated donors, since these cells have been found defective in their capacity to enhance immunization by antigens which they have taken up (3, 5, 23). Antigens labeled with ^{131}I and ^{125}I have been used to compare the fate of antigen either in different populations of peritoneal exudate cells, or in the same population exposed to antigen at different times. Pulse and chase experiments of this type (24) permit

* Present address: Institut für Genetik der Universität zu Köln, 5 Köln—Lindenthal, Germany.

minor variation in extraction and fractionation procedures to be neglected. The experiments demonstrate the existence of distinct turnover and storage compartments, and show that macrophages from irradiated donors have a defect in handling antigen related to the storage compartment.

Materials and Methods

Experimental Animals.—Male and female CBA mice, 3–9 months old, from the inbred line kept at the National Institute for Medical Research, London, were used for the experiments.

Antigens.—Heat-denatured bovine serum albumin (c BSA) was prepared according to the method of Benacerraf et al. (25). It was used as antigen in most experiments because it is taken up by peritoneal exudate cells five times as efficiently as BSA. BSA (Cohn fraction V) and bovine gamma globulin (Cohn fraction II) came from Armour. The synthetic polypeptides poly(D-Tyr, D-Glu, D-Ala)236(D-TGA), poly(L-Tyr, L-Glu)506(L-TG) were provided by Dr. Yehudit Stupp via Dr. J. H. Humphrey.

Isotope Labeling.—^{125}I and ^{131}I came from the Radiochemical Centre, Amersham, England. For the iodination of c BSA both the ICl method (26) and the chloramine-T procedure (27) were used; specific activities of the labeled proteins were 0.01–0.05 μc/μg and 1–2 μc/μg respectively. The synthetic polypeptides were labeled using the chloramine-T procedure, resulting in specific activity of 1–2 μc/μg.

Chemicals and Lysosomal Enzyme Assays.—Unless otherwise stated, chemicals were obtained from British drug houses. P-nitrophenylphosphate (Sigma 104 phosphatase substrate) and phenolphthalein glucuronic acid came from Sigma, St. Louis, Mo., Nonidet P40 from Shell Chemical Corp., New York, Triton X100 from Lenning Chemicals, London, and sodium dodecylsulfate (SDS) from Koch-Light, Colnbrook, England. Acid phosphatase and β-glucuronidase were assayed according to standard procedures (28, 29) in aliquots of the sucrose gradient fractions treated with 3% Triton X100.

Harvesting of Peritoneal Exudate Cells (PEC).—Animals were injected intraperitoneally with 3 ml thioglycolate medium (Difco) 3 days prior to harvesting of peritoneal exudate cells (PEC). Cells were harvested by injecting 4 ml chilled Gey's solution (1 unit heparin/ml) into the peritoneal cavity shortly before the mice were killed. A few minutes later an average of 3 ml of fluid containing cells were recovered. A cell yield of 5–10 $\times$ 10^6 per mouse was obtained and consisted of 70–85% macrophages.

Uptake of Antigen.—For in vivo uptake animals were injected intraperitoneally with 0.5 ml of antigen (0.1–0.5 mg/ml) in Dulbecco's phosphate buffered saline 2 hr prior to harvesting of cells. In pulse and chase experiments differently labeled antigens were given at the times described in the Results section. For in vitro uptake, harvested cells were centrifuged at 300 *g* for 10 min, and then resuspended in medium 199 at a concentration of 1 $\times$ 10^7 cells/ml. 5 ml aliquots were dispensed into polystyrene tissue culture dishes (Esco, 5 cm diameter), the cells were allowed to settle and adhere for 2 hr. The dishes were then swirled and medium and nonadherent cells sucked off, and immediately replaced by 2 ml medium 199 prewarmed to 37°C, containing radioactively labeled antigen. After 70 min the supernatant was again removed, and the dishes rinsed very gently with 2 ml prewarmed medium 199; the cells were then incubated with 2 ml prewarmed medium 199 containing the antigen with the second label for a further 5 or 20 min. Incubation was carried out at 37°C in an atmosphere of 5% CO_2 and 95% air.

Before homogenization cells were removed from the dishes with a rubber policeman and washed by centrifugation three times in cold medium 199 and then once in cold 0.25 M sucrose.

Retention of Antigen by Cells In Vitro.—Cells which had taken up antigen in vivo were washed three times by centrifugation, resuspended at a concentration of 1 $\times$ 10^7 cells/ml, and

distributed into dishes; two dishes were taken for each time point. In order to prevent adherence of cells, the dishes, containing 4 ml of cell suspension, were gently swirled during incubation. Dishes were removed at intervals; the cells were washed twice and the amount of cell-bound antigen measured.

Homogenization of Cells and Isopycnic Density Gradient Centrifugation.—Cells at a concentration of 1×10^8/ml were homogenized in 0.25 M sucrose in a smooth-walled glass tube with a tight-fitting teflon pestle (Potter-Elvehjem) at 0°C; the pestle was moved up and down by hand for 2-min periods, at intervals of 2 min in an ice bath between each period of homogenization. Phase contrast microscopy showed that 90–95% of the cells were broken when this procedure was used.

The cell homogenate was then placed on a 30–70% or 30–80% (w/v) linear sucrose gradient (29 ml), and centrifuged in a Spinco SW25 Rotor for 18 hr at 53,500 *g*. Fractions of 25 drops

TABLE I

Uptake of c BSA by PEC 2 hr after Intraperitoneal Injection into CBA Mice

Mice	μg Uptake/mg injected/10^8 cells				
Normal	3.5	8.8	2.8	1.4	mean 3.5
	0.9	2.5	1.5	4.0	
	3.2	8.8	5.5	0.19	
	6.5	1.6	3.8	1.7	
Irradiated at day −2 (900 r)	4.0	4.8	2.6	1.2	mean 2.3
	0.5	2.1	1.4	2.7	
Irradiated at day −2 (1000 r)	1.4				

each were collected from the bottom of the tube. The radioactivity and refractive index of the fractions were measured and the acid phosphatase and β-glucuronidase activity was determined. The density of the sucrose gradient fractions is expressed in the figures in terms of the refractive index, measured with an Abbé-Zeiss refractometer.

Measurement of Radioactivity.—Radioactivity due to ^{125}I and ^{131}I was measured in a Packard autogamma spectrometer under conditions where 15% of the ^{131}I counts appeared in the ^{125}I channel, and less than 1% of the ^{125}I counts in the ^{131}I channel. The data were corrected for background and overlap.

X-Irradiation of Animals.—Whole body X-irradiation was carried out at 124 kv and a dose of 37.5 r/min at a distance of 32 cm.

Immunization and Antibody Titration.—Groups of six animals were injected intraperitoneally with a priming dose of c BSA or of antigen from subcellular fractions. After 18 days they received a booster dose of 100 μg BSA intraperitoneally. The animals were bled 10 days later from the median tail artery. Titration of antibodies was carried out using a modified Farr technique.

Immunoprecipitation.—Immunoprecipitation of the supernatants of aliquots from sucrose gradient fractions was carried out according to standard procedures (31) with anti-BSA serum raised in rabbits.

RESULTS

Uptake of c BSA by Cells.—Table I shows the amount of c BSA taken up in vivo by PEC 2 hr after injection of the antigen. Animals which had not

been irradiated are compared with animals which received 900 r (or 1000 r in one case) whole body irradiation. The mean uptake for cells from nonirradiated animals is 3.5 μg/mg c BSA injected/10^8 cells and for cells from irradiated

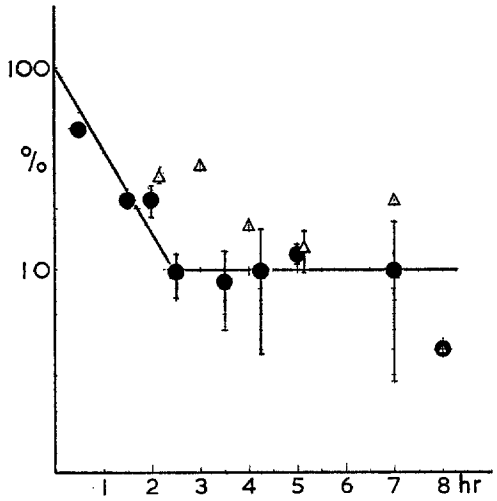

FIG. 1. Retention by cells from peritoneal exudate of antigen taken up in vivo. Abscissa: Time of incubation in vitro. Ordinate: Per cent of radioactivity retained from radioactivity at time 0. Logarithmic scale. Incubation of cells in tissue culture medium 199 (●——●). Data from 6 different experiments. Incubation in tissue culture medium 199 plus 1 mg c BSA/ml. (△——△). Two different experiments. Vertical lines indicate standard deviations.

TABLE II

Retention of c BSA after Short Pulse Phagocytosis In Vitro

Pulse	Uptake	Retained after 3 hr	
min	*cpm*	*cpm*	%
5	8,056	471	5.8
10	12,254	952	7.6
20	15,900	981	6.1

animals it is 2.3 μg/mg c BSA injected/10^8 cells. The lower uptake in the irradiated animals is not significantly different at the 5% level from the normals. The uptake in vitro (Fig. 2) is comparable to the uptake in vivo.

Fate of c BSA Taken Up by PEC In Vitro and In Vivo.—An experiment in which cells took up c BSA in vivo and were then incubated in vitro is illustrated in Fig. 1. Approximately 90% of the radioactivity initially present in the cells was released after 2–3 hr incubation, in a form not precipitable by TCA. No further release could be detected during a further 6 hr period of incubation, in which no appreciable cell loss occurred. Similar degradation

kinetics were obtained with BSA and with BGG, and have been recorded previously with hemocyanin (15). The experiment also shows that the retained fraction of antigen could not be diluted out with excess nonradioactive antigen, which implies that the retained material is segregated from the material which enters subsequently. Table II shows that after short pulse phagocytosis in vitro the final amount retained after 3 hr is in the same range as in experiments where the cells phagocytosed antigen in vivo for 2 hr. Furthermore, increasing

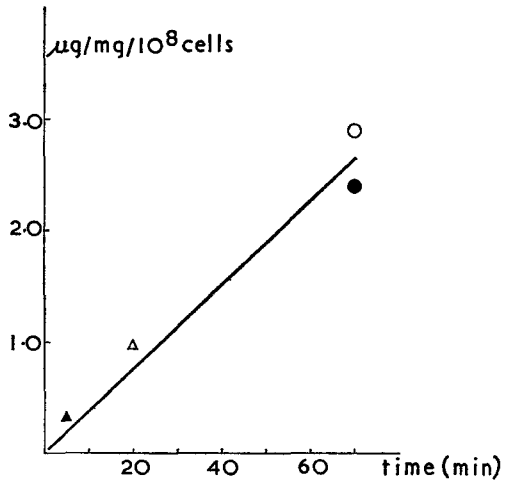

FIG. 2. Amount of c BSA taken up by cells in a pulse and chase experiment in vitro. Data for two parallel experiments (open and closed symbols) are given. Circles refer to the uptake of ^{125}I c BSA during 70 min incubation; triangles refer to the uptake of ^{131}I c BSA during 5 or 20 min. Cells are further analyzed in the experiment shown in Fig. 3.

the length of the pulse time does not affect the size of the fraction finally retained.

Demonstration of Turnover and Storage Compartments.—The following experiment was performed in order to identify the route taken by antigen after uptake. Two aliquots of cells were exposed to ^{125}I c BSA for 70 min in vitro; a chase of ^{131}I c BSA was then given for either 5 or 20 min (Fig. 2). The uptake is linearly proportional to time, confirming the impression gained from the retention data (Table II) that the experimental procedure does not harm the cells. The cells were then homogenized and the subcellular particles subjected to isopycnic centrifugation in a 30–80% sucrose gradient. For analysis of the amount of long-term *versus* chase antigen in each fraction of the gradient the ratio of $^{125}I/^{131}I$ of each fraction was determined and compared with the $^{125}I/^{131}I$ ratio of the total cell homogenate, which henceforward is called the "input ratio." A $^{125}I/^{131}I$ ratio higher than the input ratio was interpreted as

either enrichment of long-term material or deficiency in chase material and vice versa. The results are given in Fig. 3. The majority of both long-term and chase antigen is found in a fraction of density $\rho = 1.19$ g cm^{-3}, but the low ratio of $^{125}I/^{131}I$ indicates that relatively more of the antigen taken up during the 5 or 20 min phase is found in this fraction than of the antigen which was taken up during the preceding 70 min. The latter, on the other hand, appears to be enriched in a fraction of density $\rho = 1.26$ g cm^{-3} which contains only about 10% of the total radioactivity found in the gradient. The ratios of $^{125}I/$

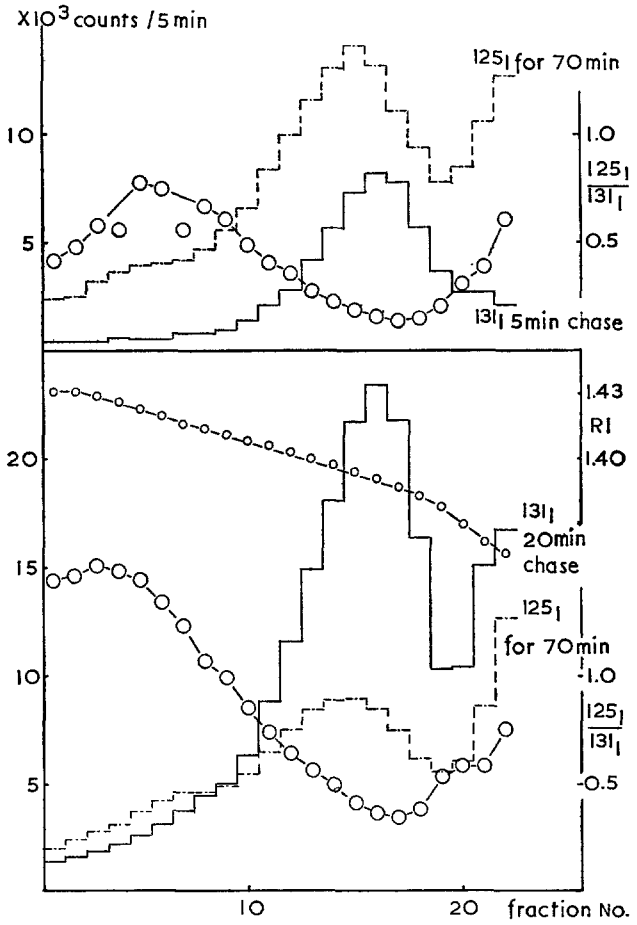

FIG. 3. Distribution of c BSA in subcellular fractions of PEC. Analysis of cell homogenates from pulse and chase experiment in Fig. 2 in a 30–80% sucrose gradient. The 5 min *versus* 70 min label is shown on the upper graph; the 20 min *versus* 70 min label on the lower graph. Fraction I is the bottom fraction of the gradient. RI is refractive index of sucrose fractions (small circles). The ratio of $^{125}I/^{131}I$ is given for each fraction (large circles).

^{131}I differ over the gradient by a maximum factor of 5.5 in the 5 min *versus* 70 min experiment, and by a maximum factor of 4.3 in the 20 min *versus* 70 min experiment. Some radioactivity was found at the top of the gradient after phagocytosis for 70 and 20 min, but not after 5 min. Approximately 50% of this radioactivity is not TCA precipitable. From this and from the time course of its appearance, the top fraction can be interpreted as intracellular degradation products which are released after homogenization.

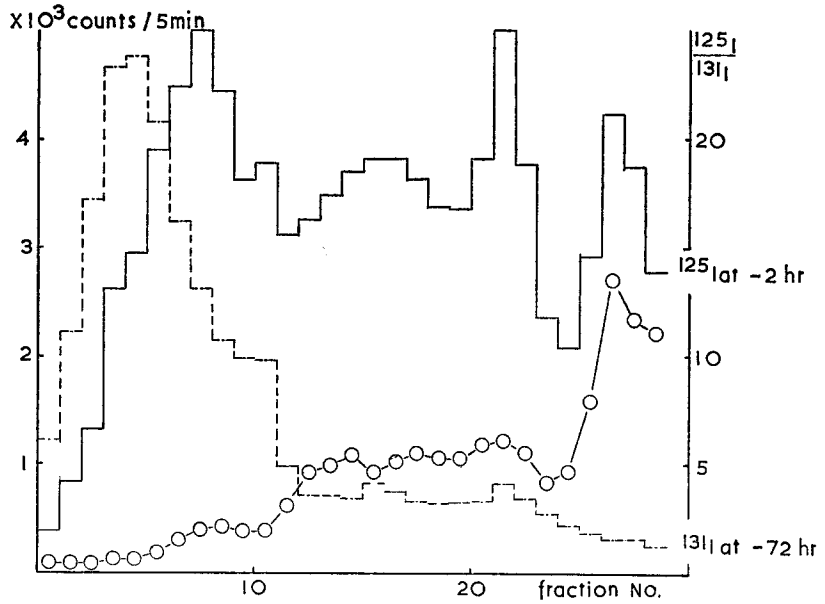

FIG. 4. Sucrose gradient analysis of a pulse and chase experiment in vivo. Distribution of radioactivity in subcellular fractions on a 30–80% sucrose gradient. ^{131}I c BSA was given at −72 hr, followed by ^{125}I c BSA at −2 hr.

In order to explore longer term retention, a similar experiment was performed in vivo. Mice were injected intraperitoneally with 3 ml thioglycolate medium at day −6, 100 μc ^{131}I c BSA at −72 hr, and 10 μc of ^{125}I c BSA at −2 hr. At time 0 hr PEC were harvested, homogenized, and analyzed in a sucrose gradient (Fig. 4). Most of the ^{131}I label persisting after 72 hr is found in the 1.26 fraction. The maximum difference in the $^{125}I/^{131}I$ ratio over the gradient is 20- to 30-fold. There is a slight discrepancy in the density distribution of the 2 hr label between this last experiment and the in vitro experiment described in Fig. 3, attributed perhaps to the difference in timing of the thioglycolate treatment. The density 1.26 storage compartment and the density 1.19 turnover compartment could still be identified.

The subcellular distribution of antigen in cells from animals which received

a dose of either 1 μg c BSA or 1000 μg, has also been compared. The input ratio of $^{125}I/^{131}I$ was 0.44 and the ratio of the fractions varied by a maximum of 11% from this value. The lack of saturation over the dose range from 1–1000 μg suggests that natural antibodies do not play a role in transferring or holding c BSA in the storage compartment.

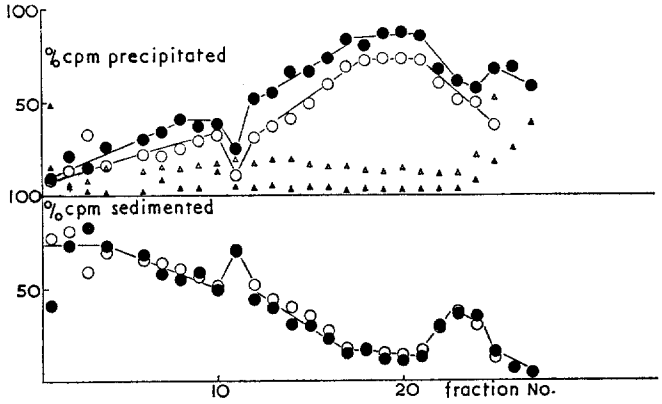

FIG. 5. Analysis of radioactive label in subcellular fractions separated on a 30–80% sucrose gradient. The fraction number is given on the abscissa. Fraction I is the bottom fraction. Bottom: aliquots of fractions (0.1 ml diluted 1:5 in borate buffer pH 8.4, 3% Triton X100) were centrifuged at 2500 *g* for 30 min. Radioactivity in sediment was determined and expressed in per cent counts per minute of total radioactivity in the aliquot. Two parallel determinations (○——○, ●——●). Top: the radioactivity in the supernatants in one of the parallel samples was precipitated with 5% TCA (final volume 0.8 ml), and TCA-precipitable (●——●) and nonprecipitable (▲——▲) radioactivity was determined. The radioactivity in the supernatants of the other parallel sample was precipitated with rabbit anti-BSA antiserum and precipitable (○——○) and nonprecipitable (△——△) radioactivity was determined. Reaction mixture consisted of 0.1 ml fraction aliquot, 0.4 ml borate buffer, 25 μg cold BSA in 0.01 ml, 0.3 ml rabbit anti-BSA antiserum 1/10 diluted, and was kept at 4°C for 15 hr. Radioactivity in precipitates was determined and expressed in per cent counts per minutes of total radioactivity in aliquot.

TABLE III

Effect of Detergents and Urea on Solubility of ^{125}I c BSA from a Gradient Fraction of ρ = 1.26 g cm^{-3}

	2500 g sediment	Supernatant
	cpm	*cpm*
0.1 ml + 0.5 ml borate buffer pH 8.4	470	110
+1% SDS	85	468
+3% Triton X100	389	192
+1% Nonidet P40	406	195
+10% Urea	378	183

Characterization of the Radioactivity Retained in the Sucrose Gradient Fractions.—Cells containing c BSA and treated with 3% Triton X100 for 30 min release 45–55% of the radioactivity. The residual radioactivity can be sedimented with cell debris by centrifugation at 2500 *g* for 30 min. The same result was found for flagella antigen in lymphoid tissues of rats (32). Among the fractions of the sucrose gradient the solubility of the radioactivity after treatment with Triton X100 varies greatly (Fig. 5). In the 1.26 region 70–80% is sedimentable, while in the 1.19 region this is only 10–20%. With the exception

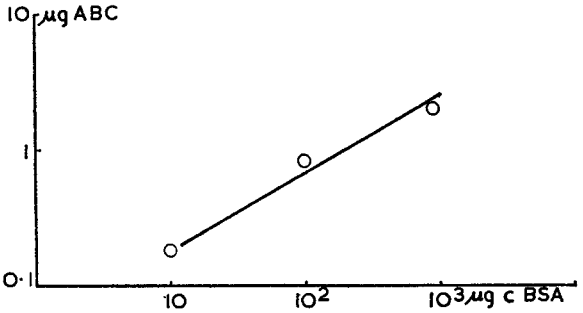

FIG. 6. Correlation between priming dose of c BSA and antibody titers 10 days after a 100 μg booster dose of BSA. Controls for experiment in Table IV.

TABLE IV

Failure to Immunize with Subcellular Fractions from PEC Containing c BSA

Pooled fractions	c BSA injection/mouse	ABC*/ml serum	Standard deviation
	μg	μg	
A			
ρ = 1.26–1.29	1	0.21	1.72
	10	0.21	1.60
	100	0.12	1.16
B			
ρ = 1.23–1.25	1	0.14	1.43
	10	0.45	2.17
	100	0.25	1.86
C			
ρ = 1.15–1.20	1	0.12	1.53
	10	0.16	1.50
	100	0.18	1.78
D			
ρ = 1.05–1.10	1	0.23	2.38
	10	0.23	1.57
	100	0.20	2.26

* Antibody-binding capacity.

of the radioactivity found in the three upper fractions of the gradient, most of the radioactivity remaining in the supernatant after centrifugation can be precipitated with 5% TCA and anti-BSA serum. Aliquots of the 1.26 fraction from a different experiment were treated with urea and several detergents. It can be seen in Table III that only SDS solubilizes the radioactivity to an appreciable extent.

The data show that it is predominantly the antigen in the storage compartment which resists solubilization, indicating a special binding which was already suggested from the "wash-out" experiments (Fig. 1).

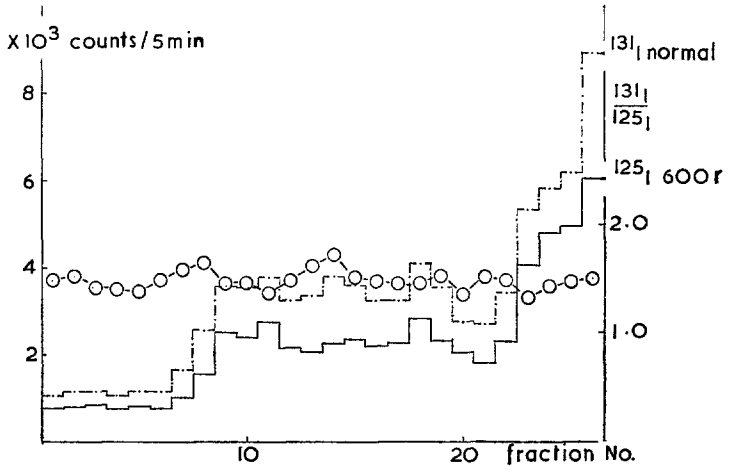

FIG. 7. Comparison of the subcellular distribution of antigen in normal cells and cells from animals irradiated with 600 r at day −2. Ratio of $^{131}I/^{125}I$ (O——O).

Attempts to Immunize Mice with Antigen from Subcellular Fractions.—We have tried to prime animals with antigen from subcellular fractions and have measured the ability of animals injected with these fractions to respond to a subsequent booster dose of 100 μg of BSA; a single injection of 100 μg BSA into normal CBA mice does not induce any detectable antibody formation (30). Fig. 6 shows the dose-response curve of control animals for a 10–1000 μg priming dose of c BSA. The experimental animals received 1, 10, or 100 μg c BSA from pooled subcellular fractions, the density range of which is included in Table IV. The amount of antigen injected was calculated on the basis of the radioactivity found in the fractions. Even a dose of 100 μg of antigen from the subcellular fractions failed to prime the animals to any significant extent. The antibody titer was within the range one would expect from controls receiving only 10–50 μg c BSA priming dose.

Further Studies on the Storage Compartment: the Effect of X-Irradiation on the

Intracellular Fate of c BSA.—The following experiments were designed to test whether any alteration in the handling of antigen by macrophages can be detected after X-irradiation of the cell donors. Animals to be used as controls received thioglycolate at day −3 and were injected with c BSA 2 hr before they were killed. Experimental animals also received thioglycolate at day −3, and were X-irradiated at day −2 (5). They received c BSA labeled with the alternative isotope 2 hr before being killed. The uptake of c BSA by the cells from irradiated donors is shown in Table I. The cells were washed separately and were pooled shortly before homogenization. The method of detection of

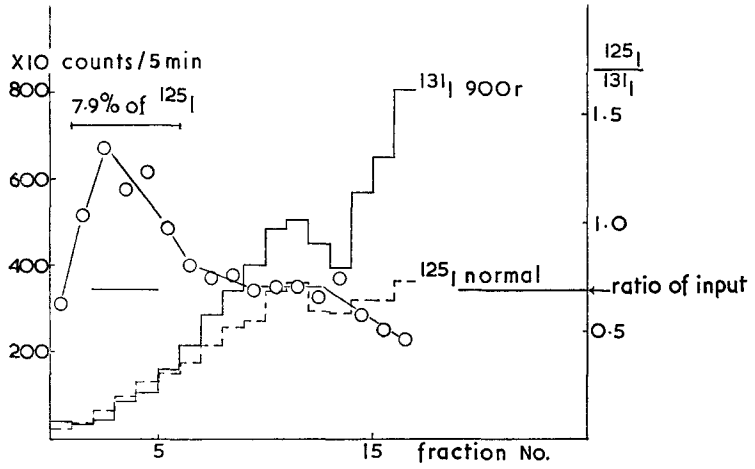

FIG. 8. Comparison of the subcellular distribution of antigen in normal cells and cells from animals receiving 900 r at day −2. Ratio of $^{125}I/^{131}I$ (O——O). See text for further details.

differences between normal and irradiated cells was again an alteration in the $^{125}I/^{131}I$ ratio within the fractions of subcellular particles from sucrose gradients. A dose of 600 r at day −2 does not alter the intracellular distribution of antigen as compared with the controls: the input ratio of $^{131}I/^{125}I$ (as it is expressed in this experiment) is 1.5, and the ratios of the single fractions lie within ±13% of that value (Fig. 7). However, preirradiation with 900 r does change the subcellular distribution of antigen (Fig. 8). In this experiment nonirradiated animals received ^{125}I c BSA, and X-irradiated animals received ^{131}I c BSA. The ratio in the fractions of the gradient varies by a factor of 3, the highest ratio of $^{125}I/^{131}I$ being found at a density of 1.26, whereas the ratio in the fractions in the 1.19 region corresponds to the input ratio. It is interesting to note that the radioactivity found in normal cells in the dense fractions in which the ratio of $^{125}I/^{131}I$ is increased amounts to 7.9%. This figure corresponds well with the 10% of antigen which is retained in the storage compartment.

An experiment similar to the one shown in Fig. 8 is shown in Fig. 9, with the labels reversed. The ratio of $^{125}I/^{131}I$ varies by a factor of 2.7 over the gradient and the lowest $^{125}I/^{131}I$ ratio is found in the 1.26 region, showing that the ratio alteration is not an artefact introduced by the particular isotopes used.

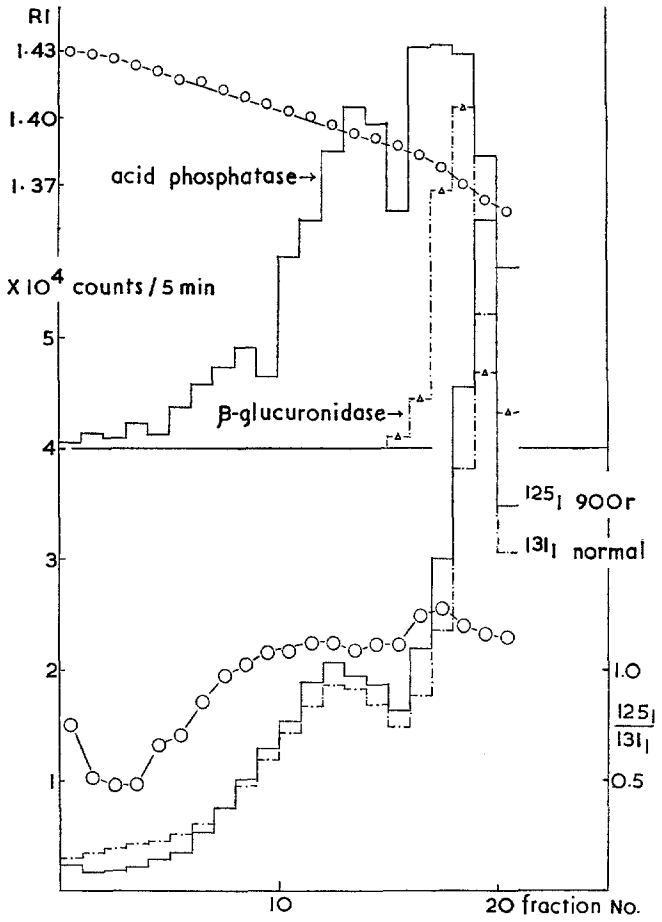

FIG. 9. Comparison of the subcellular distribution of antigen in normal cells and cells from animals exposed to 900 r at day −2. Similar to experiment in Fig. 8, but with inversion of the radioactive label. Acid phosphatase and β-glucuronidase activities of the fractions are given in arbitrary units.

Fig. 10 shows an experiment in which normal cells received ^{125}I c BSA and irradiated cells ^{131}I c BSA as in Fig. 8, but a sucrose gradient was used with the density of 1.26 close to the middle of the gradient. The high $^{125}I/^{131}I$ ratio remains associated with the 1.26 fraction. The ratio of $^{125}I/^{131}I$ varies by a factor

of 2.4 over the gradient. The experiment demonstrates that the ratio alteration studied is associated with a 1.26 fraction, and not merely with fast sedimentation. As in the other experiments comparing normal and irradiated cells, the

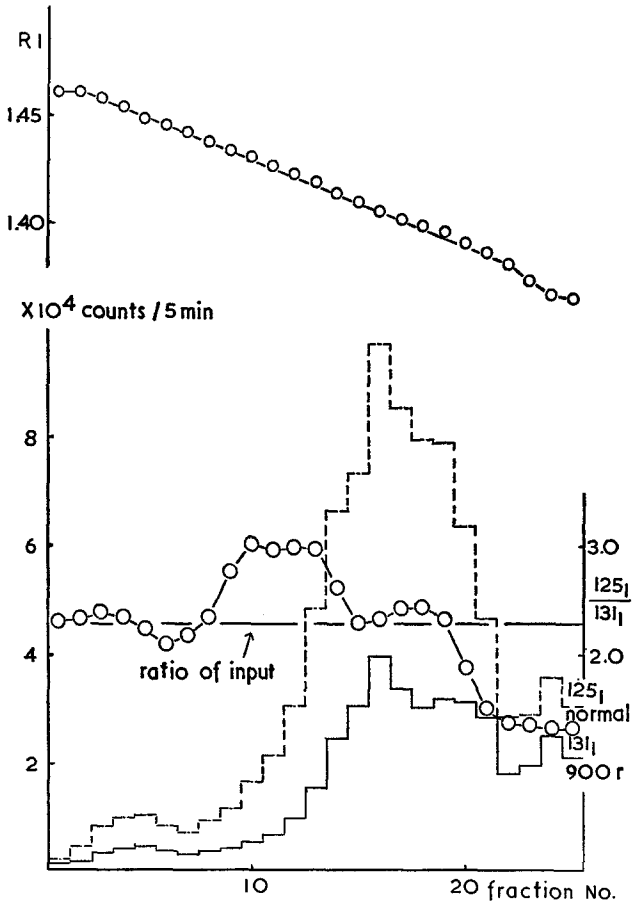

FIG. 10. The subcellular distribution of antigen from normal animals and animals irradiated with 900 r at day −2, analyzed on a 30–80% sucrose gradient. Ratio of $^{125}I/^{131}I$ (O——O).

distribution in the 1.19 region seems to be unaffected by irradiation. All three experiments show more radioactivity from irradiated cells in the top fractions of the gradient. No marked difference has been found between normal and irradiated cells in the amount of TCA-precipitable material from this fraction. Experiments similar to those done with c BSA have been repeated with bovine gamma globulin. The majority of the radioactive label was again found in the

1.19 fraction, and 5–10% on the 1.26 fraction, where after X-irradiation less label was again found than in normal cells. The data show that normal cells have relatively more radioactivity in the 1.26 fraction than do cells irradiated with 900 r 2 days before antigen is given.

Characterization of the Subcellular Fractions: Distribution of Lysosomal Enzymes.—Fig. 9 shows the distribution of acid phosphatase and β-glucuronidase within a sucrose gradient. Acid phosphatase is distributed under two major peaks of densities: 1.15 and 1.19. β-Glucuronidase is only found in the 1.15 fraction. This restriction of β-glucuronidase has not been found in lymph node (20) or spleen homogenates (33).

Some acid phosphatase activity always appears in the 1.26 fractions. In the experiment in Fig. 9 there are only trance amounts; in experiments not described in detail here up to 20% of the enzyme activity has been found as a peak in the 1.26 fraction. In this case, β-glucuronidase was also found. The distribution of the lysosomal enzymes in the 1.26 fraction showed no consistent pattern, whereas their distribution in the 1.19 and 1.15 fractions was consistent throughout the experiments.

The 1.26 storage compartment is associated in the sucrose gradient with the nuclear fraction but it also contains vesicles visible under the electron microscope, so it is by no means a homogeneous fraction. However, it cannot be composed of a small fraction of unbroken cells since after treatment with Triton X100 much less c BSA is released from the 1.26 fraction than from whole cells.

The Intracellular Fate of D-TGA and L-TG after Uptake by PEC.—D-TGA is a synthetic polypeptide with defined immunological and metabolic properties (11, 12). Its chief metabolic characteristic is that it is degraded very slowly in the body. Table V shows the retention of D-TGA and L-TG by PEC. Their retention is compared with c BSA, the numbers for which are taken from Fig. 1. After 4 hr "wash-out" in vitro 71% of D-TGA is still retained by the cells, and after 5 hr 47% of L-TG is still retained; at the same time only the final 10% of c BSA is left in the cells. It was therefore interesting to study the distribution of D-TGA and L-TG in subcellular fractions.

A group of animals received ^{125}I D-TGA intraperitoneally 2 hr before being killed. To a control group ^{131}I c BSA was given at the same time. The PEC from the two groups were harvested, washed separately, and mixed shortly before homogenization. The distribution of the c BSA is the usual one with material in the 1.19 and 1.26 fraction. D-TGA in addition to being present in the 1.26 fraction is found mostly in a fraction with density 1.15, with some radioactivity in the 1.19 region (Fig. 11). (In a separate control experiment performed with free D-TGA up to 90% of the material could be recovered from the top two fractions of the gradient. Free D-TGA does not move into the 1.15 region.)

TABLE V

Retention of D-TGA and L-TG in Macrophages In Vitro

Time	% Retained		
	D-TGA	L-TG	c BSA
hr			
0	100	100	100
2	74	60	22
3	—*	49	10
4	71	—*	10
5	—*	47	10

Data for D-TGA and L-TG are mean values from two experiments each. Data for c BSA are for comparison and are taken from Fig. 1.

* Not determined.

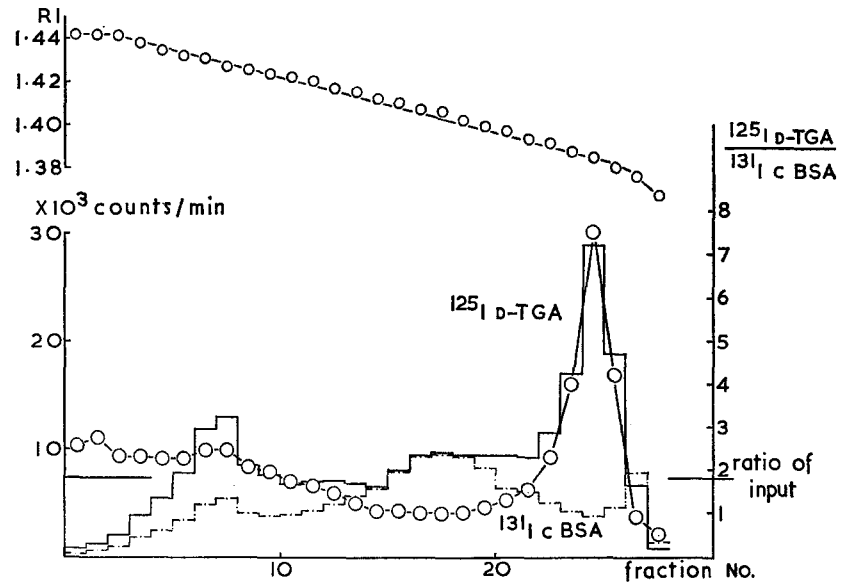

FIG. 11. Analysis of the subcellular distribution of ^{125}I D-TGA, with the distribution of ^{131}I-c BSA as a control.

In a pulse and chase experiment in vitro similar to the one described for c BSA in Fig. 2, the intracellular fate of D-TGA was further analyzed (Fig. 12). Aliquots of cells adhering to glass were allowed to take up ^{125}I D-TGA for 70 min. Then free ^{125}I D-TGA was removed with the supernatant and after rinsing, ^{131}I D-TGA containing medium was given to the cells for 5 or 20 min respectively. The analysis of the distribution of ^{125}I long-term label and the chase ^{131}I D-TGA label by comparing the ^{125}I/^{131}I ratio in the subcellular fractions in

the sucrose gradient showed three interesting features: (*a*) long-term material is enriched in the 1.26 fraction; (*b*) more long-term label than chase label is found in the 1.19 fraction, though the total amount of radioactivity is still

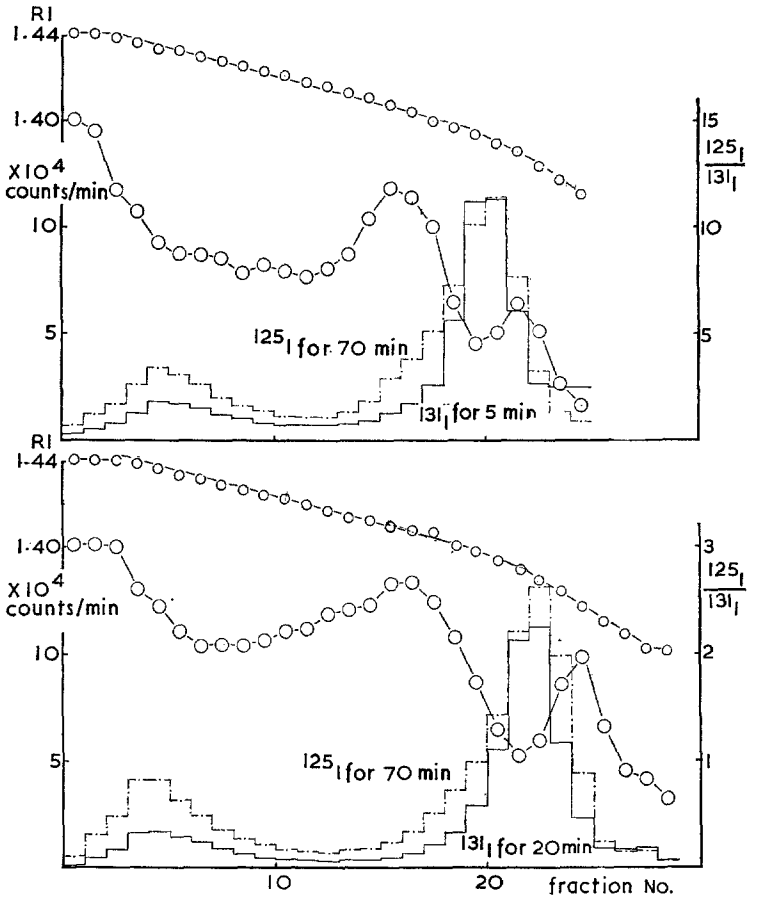

FIG. 12. Sucrose gradient analysis of a pulse and chase experiment similar to the one in Fig. 3, with D-TGA as the antigen studied. Ratio $^{125}I/^{131}I$ (large circles); refractive index (small circles).

small compared to the amount in the 1.15 fraction; (*c*) "short-term" material used for the chase is found preferentially in the 1.15 region.

In another experiment the subcellular distribution of ^{125}I D-TGA was compared with the synthetic polypeptide ^{131}I L-TG. Cells from two different groups of animals injected with the two polypeptides, were pooled shortly before homogenization. Both polypeptides were mainly retained in the 1.15 fraction;

the input ratio of $^{125}I/^{131}I$ was 3.09 and the deviation from this value among the single fractions was within ±6%.

The experiments described in this section demonstrate that the 1.15 fraction is the compartment which contains the bulk of cell-associated polypeptide material, and that the 1.26 storage compartment exists for proteins and polypeptides.

DISCUSSION

The interpretation of results obtained by the methods employed in the study are open to question in several ways. The validity of an external iodine label for antigen has been discussed in previous work (34, 35). The objection is largely answered here by the observation that the radioactivity in the cell fractions of critical interest can be precipitated, with fair efficiency, by specific antiserum. A second question concerns the distribution of antigen between cells. Some of the observed differences in localization may be attributable to cellular heterogeneity, although previous work with hemocyanin has shown that the majority of peritoneal macrophages retain the foreign protein (15). Differential susceptibility to homogenization might also be invoked to account for the observed differences, but the consistency found after different pretreatments of the cells does not support this possibility.

The 1.19 density compartment has the density and acid phosphatase content characteristic of lysosomes. During phagocytosis 90% of c BSA, BSA, or BGG are found in this compartment. The antigens are not retained there for long, but are degraded with a half-life of less than 1 hr. In this respect the present work confirms previous findings with other proteins (14, 15). The synthetic polypeptides localized in a lysosomal fraction of a density 1.15. This adds a further example of functional heterogeneity of lysosomal particles of different density from those already described (17, 36). The difference can hardly be attributed merely to size, since the polypeptides have a molecular weight about one-third that of BSA. The possibility that the 1.15 and 1.19 compartments may belong to different cells must be kept in mind (17), although there is no doubt that the cells which take up D-TGA are macrophages (11). The observation that at least some of the D-TGA reaches the 1.19 compartment after prolonged phagocytosis might argue that the 1.15 compartment lies in the pathway to the 1.19 compartment. According to this interpretation D-TGA simply demonstrates an intermediate step in the phagocytosis of proteins.

Storage of antigen apparently occurs in a separate compartment, of density 1.26. The nature of this compartment is not clear. It is associated with nuclei, and previous work has shown that antigens become associated in this way. Bowers and de Duve (17) found dextran in the nuclear fraction after uptake by spleen cells, and the amount of flagela in the nuclear fraction of rat lymph node or spleen cells slowly increases from 15 to 25% over a period of 1 day to 2

months after injection (37). Lysosomes have been found in nuclear fractions (17), and lysosomal enzymes were found during the present work in the storage compartment to a variable extent. However, the antigen in the storage compartment did not behave as though enclosed in lysosomal vesicles: it is more strongly bound to particles than is the antigen in the 1.19 lysosomal fraction, and most of it is irreversibly attached and protected from degradation.

Two lines of evidence suggest that the 90% of antigen in the lysosomal compartment is not directly involved in immunization. Proteins in this compartment have a short half-life and are degraded into amino acids (14); for hemocyanin it has been shown that only 10% of antigen taken up by macrophages is immunogenic (15), which is in agreement with the present figures for distribution of antigen between turnover and storage compartment. Immunogenicity of antigen taken up by macrophages persists (15) and in our experiments the retained antigen is found in the storage compartment. Therefore, it is possible that the deficiency in the storage compartment of X-irradiated macrophages is related to the incapacity of these cells to mediate an immune response (3, 5, 23). The loss of this macrophage function might be due to damage to the process which brings antigen into the storage compartment or retains it there for the induction of an immune response. An alternative explanation for the deficiency in the storage compartment is that X-irradiation causes labilization of this compartment towards mechanical shearing during homogenization.

Macrophage-enclosed BSA has greater immunogenic capacity in syngeneic than in allogeneic transfers. Furthermore, heat inactivation of the cells abolishes the effect, thus suggesting that the cooperation of the living cell is needed (5). It is consistent with these data that none of the subcellular fractions tested in immunization experiments showed any enhanced immunogenicity.

The observation that antigens can be located in different compartments should be considered in connection with the three functions possibly performed by macrophages. (*a*) They handle antigen and retain it in an immunogenic state (5, 13, 15, 22); (*b*) they are efficient and fast transporters of antigen through the body (38, 39); (*c*) they remove free antigen from the circulation (11, 25). If the action of antigen is really a function of macrophage intervention, then the immunogenicity of an antigen is not only dependent on how much is stored or processed in an appropriate form and transported to antigen sensitive cells, but also on the amount and speed with which potentially tolerogenic material is degraded. Thus the macrophage would contribute to this balancing mechanism. One place for degradation is the 1.19 lysosomal compartment with the 1.15 compartment playing a subsidiary role. Antigens may be in the 1.15 compartment because they resist degradation to a greater extent than does c BSA, or alternatively these antigens may resist degradation to a greater extent because they are not transported into the 1.19 turnover compartment. The combined evidence of studies on the metabolic fate of D-TGA in this and a

previous study (11) makes it seem likely that the 1.15 compartment can excrete undegraded antigen into the tolerogenic pool. In this context, it is clear that elucidation of the roles of the 1.19 and 1.15 compartments will be important.

SUMMARY

The intracellular fate of phagocytosed antigens in cells from peritoneal exudate in CBA mice has been studied by using ^{125}I and ^{131}I labeled antigens. After uptake of labeled antigen, cells were homogenized and the subcellular fractions were analyzed by isopycnic centrifugation in a sucrose gradient.

The uptake of heat-denatured BSA (c BSA) by these cells in vivo is 3.5 μg/mg c BSA injected/10^8 cells. The uptake by cells in animals which were exposed 2 days earlier to 900 r whole body irradiation is slightly lower but does not differ significantly. 90% of the phagocytosed material is degraded within 2–3 hr, the residual 10% is retained at least over an 8 hr periods. Using a pulse and chase technique, with ^{125}I and ^{131}I c BSA in vitro and in vivo it was shown that newly phagocytosed antigen is found mainly in a lysosomal turnover compartment of a density 1.19 g cm^{-3}. Antigen which has been in the cells for longer was found in a denser fraction (1.26 g cm^{-3}). In a comparison of nhrmal and X-irradiated cells it can be shown that after irradiation with 900 r less c BSA is found in this storage compartment.

Binding of the antigen to the subcellular fractions, and its behavior towards several detergents has been studied. Subcellular fractions do not have the increased immunogenic capacity of antigen enclosed in living macrophages.

Two synthetic polypeptide antigens, poly(D-Tyr, D-Glu, D-Ala) and poly-(L-Tyr, L-Glu) have a different subcellular distribution from c BSA, BSA, or bovine gamma globulin. Apart from also being found in the 1.26 storage compartment the polypeptide antigens are mainly located in a 1.15 compartment and only to a small extent in the 1.19 compartment. The half-life of these antigens in the cells is much longer than the half-life of the protein antigens studied.

The finding of several subcellular compartments is discussed in connection with the functions possibly performed by macrophages.

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BIBLIOGRAPHY

1. Leskowitz, S. 1967. Tolerance. *Ann. Rev. Microbiol.* **21**:157.
2. Dresser, D. W., and N. A. Mitchison. 1968. The mechanism of immunological paralysis. *Advan. Immunol.* **8**:129.
3. Pribnow, J. F., and M. S. Silverman. 1967. Studies on the radiosensitive phase of the primary antibody response in rabbits. I. The role of macrophages. *J. Immunol.* **98**:225.

4. Mitchison, N. A. 1967. Immunological tolerance. *J. Clin. Path.* **20**:451.
5. Mitchison, N. A. 1968. The immunogenic capacity of antigen taken up by peritoneal exudate cells. *Immunology*. In press.
6. Unanue, E., and B. A. Askonas. 1968. *Immunology*. In press.
7. Unanue, E., and B. A. Askonas. 1967. Two functions of macrophages and their role in the immune response. *J. Reticuloendothelial Soc.* **4**:440.
8. Boak, J. L., E. Kölsch, and N. A. Mitchison. 1968. Immunological tolerance and inhibition by hapten. *In* The Immune Response and Its Suppression. E. Sorkin, editor. In press.
9. Felton, L. D., B. Prescott, G. Kauffmann, and B. Ottinger. 1955. Pneumococcal antigenic polysaccharide substances from animal tissues. *J. Immunol.* **74**:205.
10. Coons, A. H. 1963. Tolerance and persistence of antigen. *In* La tolérance acquise et la tolérance naturelle à l'égard de substances antigéniques définies. A. Bussard, editor. Editions du Centre National de la Recherche Scientifique, Paris. 121.
11. Janeway, C. A. and J. H. Humphrey. 1968. Synthetic antigens composed exclusively of L- or D-amino acids. II. Effect of optical configuration on the metabolism and fate of synthetic polypeptide antigens in mice. *Immunology*. **14**:225.
12. Janeway, C. A., and M. Sela. 1967. Synthetic antigens composed exclusively of L- or D-amino acids. I. Effect of optical configuration on the immunogenicity of synthetic polypeptides in mice. *Immunology*. **13**:29.
13. Cohn, Z. A. 1962. Influence of rabbit polymorphonuclear leucocytes and macrophages on the immunogenicity of *Escherichia coli*. *Nature*. **196**:1066.
14. Ehrenreich, B. A., and Z. A. Cohn. 1967. The uptake and digestion of iodinated human serum albumin by macrophages *in vitro*. *J. Exptl. Med.* **126**:941.
15. Unanue, E. R., and B. A. Askonas. 1968. Persistence of immunogenicity of antigen after uptake by macrophages. *J. Exptl. Med.* **127**:915.
16. Nossal, G. J. V., A. Abbot, and J. Mitchell. 1968. Antigens in immunity. XIV. Electron microscopic radioautographic studies of antigen capture in the lymph node medulla. *J. Exptl. Med.* **127**:263.
17. Bowers, W. E., and C. de Duve. 1967. Lysosomes in lymphoid tissues. III. Influence of various treatments of the animals on the distribution of acid hydrolases. *J. Cell Biol.* **32**:349.
18. Franzl, R. E. 1962. Immunogenic sub-cellular particles obtained from spleens of antigen-injected mice. *Nature*. **195**:457.
19. Uhr, J. W., and G. Weissmann. 1965. Intracellular distribution and degradation of bacteriophage in mammalian tissues. *J. Immunol.* **94**:544.
20. Williams, J. M., and G. L. Ada. 1967. Antigens in tissues. III. The separation of antigen-containing components from lymphoid tissues. *Immunology*. **13**:249.
21. Mitchison, N. A. 1968. Antigen recognition responsible for the induction *in vitro* of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32**:431.
22. Fishman, M., and F. L. Adler. 1968. The role of macrophage-RNA in the immune response. *Cold Spring Harbor Symp. Quant. Biol.* **32**:343.
23. Gallily, R., and M. Feldman. 1967. The role of macrophages in the induction of antibody in X-irradiated animals. *Immunology*. **12**:197.

24. Dintzis, H. M. 1961. Assembly of the peptide chains of hemoglobin. *Proc. Nat. Acad. Sci.* **47**:247.
25. Benacerraf, B., G. Biozzi, B. N. Halpern, C. Stiffel, and D. Mouton. 1957. Phagocytosis of heat denatured human serum albumin labelled with ^{131}I and its use as a means of investigating liver blood flow. *Brit. J. Exptl. Path.* **38**:35.
26. McFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. *Nature.* **182**:53.
27. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labelled growth hormone of high specific radioactivity. *Biochem. J.* **89**:114.
28. Schmidt, G. 1955. Acid prostatic phosphatase. *Methods Enzymol.* **2**:523.
29. Bowers, W. E., J. T. Finkenstaedt, and C. de Duve. 1967. Lysosomes in lymphoid tissue. I. The measurement of hydrolytic activities in whole homogenates. *J. Cell. Biol.* **32**:325.
30. Mitchison, N. A. 1964. Induction of immunological paralysis in two zones of dosage. *Proc. Roy. Soc. (London), Ser. B.* **161**:275.
31. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry. 2nd Edition. C. C Thomas, Springfield.
32. Ada, G. L., and J. M. Williams. 1966. Antigens in tissues. I. State of bacterial flagella in lymph nodes of rats injected with isotopically-labelled flagella. *Immunology.* **10**:417.
33. Bowers, W. E., and C. de Duve. 1967. Lysosomes in lymphoid tissue. II. Intracellular distribution of acid hydrolases. *J. Cell. Biol.* **32**:339.
34. Walter, H., S. Fleischer, and F. Haurowitz. 1961. Effect of different external labels on the metabolism of doubly labelled heterologous proteins. *Arch. Biochem. Biophys.* **95**:290.
35. Humphrey, J. H., B. A. Askonas, I. Auzins, I. Schechter, and M. Sela. 1967. The localization of antigen in lymph nodes and its relation to specific antibody-producing cells. II. Comparison of iodine-125 and tritium labels. *Immunology.* **13**:71.
36. Filipovic, I., and E. Buddecke. 1968. Wechselseitige Aktivierung und intrazelluläre Lokalisation von Hyaluronidase und Kathepsin D aus Rindermilz. *Hoppe-Seyler's Z. Physiol. Chem.* **349**:533.
37. Ada, G. L. 1967. Specialized cell function in the lymphoid and reticuloendothelial cell series. *In* Immunity, Cancer and Chemotherapy. Basic Relationships on the Cellular Level. E. Mihic, editor. Academic Press, New York. 17.
38. Roser, B. 1965. The distribution of intravenously injected peritoneal macrophages in the mouse. *Australian J. Exptl. Biol. Med. Sci.* **43**:553.
39. Kölsch, E. 1968. Migration of macrophages carrying antigen into the thymus. *Experientia.* **24**:951.