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**Biochemical Analysis of the Pinocytotic Process, III**[1, 2]

# Subcellular Distribution and Metabolic Effects of [$^3$H]Triton WR-1339

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*Dedicated to Prof. E. Klenk on the occasion of his 75. birthday*

**Summary:** The distribution of [$^3$H]Triton WR-1339 in the rat liver cell was studied between 1 and 96 h after injection. In serum and liver cell fractions of [$^3$H]Triton treated animals and in serum incubated *in vitro* with [$^3$H]Triton a labelled band with an electrophoretic mobility identical in all samples was detected. This suggests binding of the label to a protein. After injection of a high dose (175 mg/100 g rat) Triton accumulates in the plasma membrane and in the cell sap with a maximum around 24 after injection. The accumulation of Triton in the lysosomal system leads to a decreasing density of the lysosomes. The maximum uptake in a heavy lysosomal fraction (density $d = 1.13–1.18$) precedes that of a light lysosomal fraction ($d = 1.12$) indicating the heavy tritosome to be a precursor organelle of the light tritosome.
After a low dose (5 mg/100 g rat) of Triton there is no accumulation in the cell sap after 24 h and no light tritosomes are formed. An increase of lysosomal enzymes in the cell sap is only observed after the high dose. The synthesis of cellular membranes is strongly depressed under the influence of Triton. It is concluded that the metabolic derangement after the high dose of Triton leads to a process analogous to active autophagocytosis. Therefore tritosomes may arise from a combined pinocytosis and autophagocytosis.

**Zusammenfassung:** *Biochemische Analyse pinozytotischer Vorgänge III: Subzelluläre Verteilung und Stoffwechselwirkung von [$^3$H]Triton WR-1339.* Die Verteilung von Triton WR-1339 in der Leberzelle wurde mit $^3$H-markiertem Triton zwischen 1 und 96 h nach Injektion untersucht. Das nichtionische Detergenz war im Blutserum und in verschiedenen Zellfraktionen mit geringer, aber gleichartiger elektrophoretischer Beweglichkeit nachweisbar. Triton akkumuliert nach einer hohen Dosis (175 mg/100 g Ratte) zuerst in der Plasmamembran und im löslichen Zellanteil mit einem

*Abbreviations:* Tritosomes = Triton filled lysosomes; U = international unit for enzyme activities = μmol substrate/min.

*Enzymes:*

Acid phosphatase, orthophosphoric monoester phosphohydrolase (EC 3.1.3.2)
cytochrome oxidase, ferrocytochrome *c*: oxygen oxidoreductase (EC 1.9.3.1)
glucose-6-phosphatase, D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9)
glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate: NADP oxidoreductase (EC 1.1.1.49)
β-glucuronidase, β-D-glucuronide glucuronohydrolase (EC 3.2.1.31)
5′-nucleotidase, 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5).

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Maximum 24 h nach der Injektion. Mit der Aufnahme von Triton in Lysosomen nimmt deren Dichte ab. Die maximale Markierung einer schweren Tritosomen-Fraktion (Dichte $d = 1.13-1.18$) geht der einer leichten Tritosomen-Fraktion voraus. Daraus wird geschlossen, daß die schweren Tritosomen als Vorstufe der leichten Tritosomen zu betrachten sind.

Nach Verabreichung von wenig Triton (5 mg/100 g Ratte) ist keine Anreicherung von Radioaktivität in der löslichen Fraktion zu beobachten. Es werden keine leichten Tritosomen gebildet.

Ein deutlicher Anstieg lysosomaler Enzyme in der löslichen Fraktion wird nur nach der hohen Tritondosis beobachtet. Die zelluläre Membransynthese ist unter dem Einfluß von Triton stark beeinträchtigt.

Es wird daraus geschlossen, daß die schwere Zellstoffwechselstörung zu intrazellulärer Autophagozytose führt und daß bei der Bildung sekundärer, tritongefüllter Lysosomen, den sogenannten Tritosomen, sowohl Pinozytose als auch Autophagozytose beteiligt sind.

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In previous studies[1,2] the contribution of the plasma membrane to the formation of the secondary lysosomal membrane during pinocytosis was investigated using Triton WR-1339 as a model substance. It was found that neither the chemical composition nor the enzymic properties supported a direct relationship of these two membranes. In order to analyze the formation of secondary lysosomes in more detail the present report deals with the uptake and distribution as well as some metabolic side effects of [$^3$H]Triton WR-1339 in the rat liver cell.

## Results

### *Dosage of [$^3$H-]Triton*

The uptake of Triton WR-1339 by the rat liver was studied by cell fractionation after intravenous administration of $^3$H-labelled Triton WR-1339. In most experiments 350 mg of [$^3$H]Triton (specific activity: $8.2 \times 10^5$ dpm/mg, total activity $2.88 \times 10^8$ dpm) was injected intravenously into rats weighing about 200 g. This dose is equivalent to that used in the procedure originally introduced by WATTIAUX *et al.*[3] In order to study the effect of the Triton dose on its uptake by the liver in some experiments 10 mg of [$^3$H]Triton WR-1339 were administered (spec. activity: $2.88 \times 10^7$ dpm/mg, total activity $2.88 \times 10^8$ dpm).

### *Binding of Triton to serum proteins*

After injection as well as after *in vitro* incubation [$^3$H]Triton was found to be bound to a serum component with a low electrophoretic mobility, most probably a protein. This could be demonstrated by paper-electrophoresis and by polyacrylamide gel disc-electrophoresis in alkaline pH-systems.

The binding of Triton to protein also occurred in the cell sap as well as in the tritosomes. The electrophoretic mobility of the bound Triton found in the cell sap and in the lysosomes proved to be identical with that of the serum as shown by electrophoreses of mixed extra- and intracellular samples (Fig. 1). The nonionic detergent Triton WR-1339 alone exhibited no electrophoretic mobility but migrated only following the addition of serum protein.

### *Disappearance of [$^3$H]Triton WR-1339 from the blood and its uptake by the liver*

The disappearance of [$^3$H]Triton from the blood is shown in Fig. 2. 35fold dilution of 10 mg of [$^3$H]Triton WR-1339 with inactive Triton WR-1339 reduces the specific activity of serum portions to about half the value. This indicates the overloading of the binding capacity of the serum.

The total uptake of Triton by the liver (dpm/mg of protein of homogenate) exhibits the following characteristics dependent on the Triton dose applied:

Administration of a small Triton dose (10 mg) results in a normal saturation curve whereas a high dose (350 mg) leads to an irregular curve particularly between 24 and 48 h (Fig. 3).

### *Distribution of [$^3$H]Triton within the cell*

Two cell fractionation schemes were used (see Fig. 9) to follow the Triton distribution within the

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[1] I. Commun.: R. HENNING, H. D. KAULEN and W. STOFFEL, this Journal **351**, 1191 [1970].

[2] II. Commun.: H. D. KAULEN, R. HENNING and W. STOFFEL, this Journal **351**, 1555 [1970].

[3] R. WATTIAUX, M. WIBO and P. BAUDHUIN, in G. E. WOLSTENHOLME and C. M. O'CONNOR: A Ciba Foundation Sympos. on Lysosomes, p. 176, J. A. CHURCHILL, London 1963.

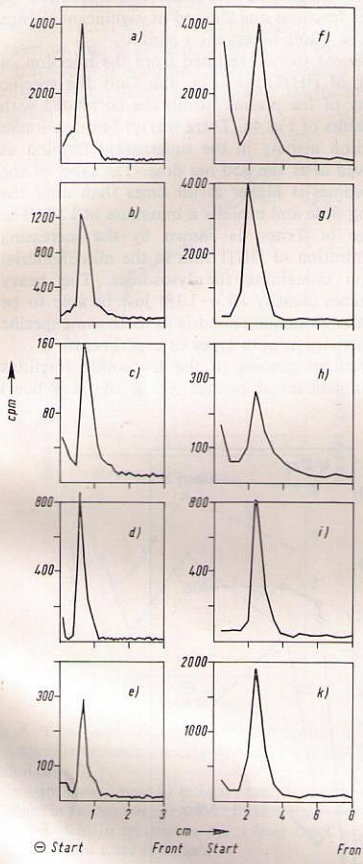


Fig. 1. Electrophoretic analysis of $^3$H-radioactivity in cell fractions and serum.

a)—e) Polyacrylamide gel disc electrophoresis.

f)—k) Cellulose acetate electrophoresis.

a) 2 μg [$^3$H]Triton WR-1339 incubated *in vitro* with 50 μl of serum

b) 100 μg of serum protein (1 h)

c) 100 μg of cell sap protein (96 h)

d) 27 μg tritosomal protein (48 h)

e) 10 μg of serum protein mixed with 100 μg of cell sap protein

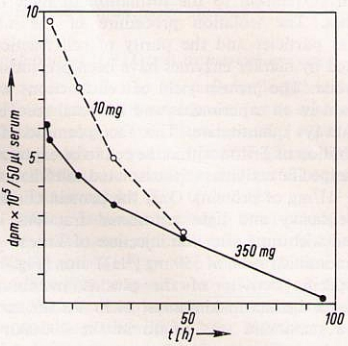


Fig. 2. Elimination of [$^3$H]Triton WR-1339 from blood serum.

○---○: Injection of 10 mg [$^3$H]Triton (spec. activity: $2.88 \times 10^7$ dpm/mg) total $2.88 \times 10^8$ dpm); ●——●: injection of 350 mg [$^3$H]Triton WR-1339 (spec. activity $8.2 \times 10^5$ dpm/mg, total activity: $2.88 \times 10^8$ dpm) ($n=3$); abscissa: time after [$^3$H]Triton injection.

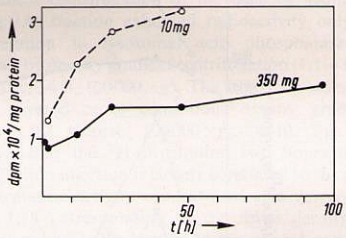


Fig. 3. Total uptake of [$^3$H]Triton WR-1339 by liver (dpm/mg of liver homogenate protein).

Spec. activities as in Fig. 2. ○---○: 10 mg [$^3$H]Triton WR-1339 dose; ●——●: 350 mg [$^3$H]Triton WR-1339 dose. Specific and total activities as in Fig. 2 ($n = 3$); abscissa: time after injection.

f) same conditions as in a)

g) 5 mg of serum protein (2 h)

h) 175 μg of cell sap protein (24 h)

i) 5 μg tritosomal protein (96 h)

k) 6 μg tritosomal protein (96 h) mixed with 185 μg of cell sap protein (2 h).

Values in parentheses give the time after [$^3$H]Triton WR-1339 injection.

cell in correlation to the formation of light tritosomes. The isolation procedure of the subcellular particles and the purity of cell fractions checked by marker enzymes have been previously[1] described. The protein yield of cell fractions was constant in all experiments and the total recovery was always quantitative. This fact permitted the distribution of Triton within the cell to be expressed by the specific activities of the isolated cell fractions (dpm $^3$H/mg of protein). Only the protein content of the heavy and light tritosomal fractions increased with time after the injection of Triton.

After administration of 350 mg [$^3$H]Triton (Fig. 4a) the specific activity of the plasma membrane showed a distinct maximum at 24 h. At the same time a maximum was found in the 100000 $\times g$ supernatant. The steady increase of radioactivity in the light tritosomes reflects the accumulation of Triton in this fraction. The radioactivity present in mitochondria and microsomes was the lowest of the cell fractions and showed no significant change over the whole observation period.

A different picture resulted from the injection of 10 mg of [$^3$H]Triton (Fig. 4b). Only the specific activity of the plasma membrane correlated with the results of Fig. 4a. There was no 24-h-maximum of Triton activity in the supernatant fraction as observed after the 350 mg dose. The label of the microsomes is higher at all times than after the 350 mg dose and exhibits a minimum at 12 h. The storage of Triton is shown by the increasing concentration of [$^3$H]Triton in the mitochondrial fraction containing the lysosomes. The heavy tritosomes (density 1.13—1.18) just be able to be separated from mitochondria have the same specific radioactivity in both types of experiments.

The protein content of the tritosomal fractions (mg of tritosomal protein per g of fresh liver)

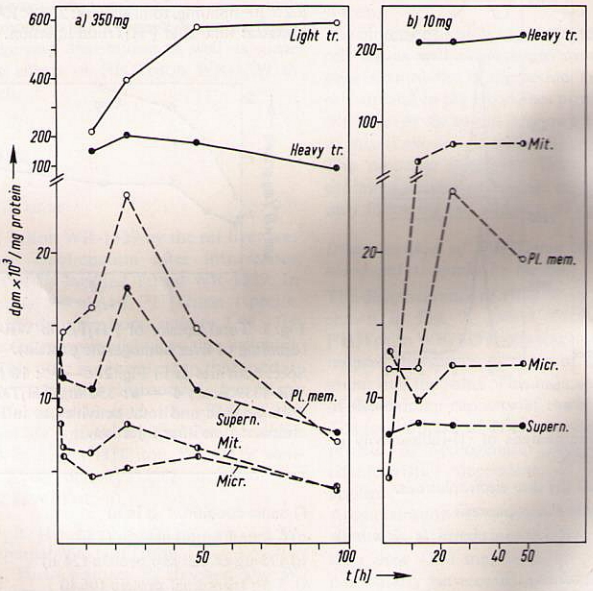


Fig. 4. Specific activities of cell fractions ($n = 3$).

a) 350 mg [$^3$H]Triton dose; b) 10 mg [$^3$H]Triton dose; (spec. activities as in Fig. 2). ○——○: Light tritosomes; ●——●: heavy tritosomes; ○---○: plasma membranes; ●---●: cell sap (supernatant); ◑---◑: mitochondria; ◐---◐: microsomes; abscissae: time after injection.

increases with the progressive uptake of Triton WR-1339. This relation between tritosome formation and Triton WR-1339 accumulation is demonstrated in Fig. 5. Both parameters, the total yield of tritosomal protein and tritosomal-bound [$^3$H]-Triton are correlated with the liver weight in this diagram.

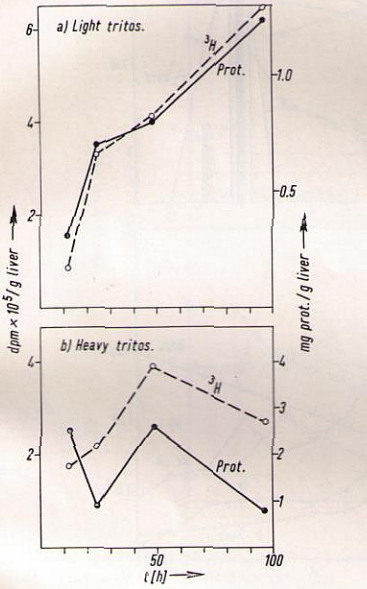


Fig. 5. Total yield of radioactivity and protein in light and heavy tritosomes after injection of 350 mg of [$^3$H]-Triton ($n = 3$).
a) In light tritosomes; b) in heavy tritosomes. ○---○: $^3$H dpm in tritosomes per g liver fresh weight; ●——●: mg of protein in tritosomes per g liver fresh weight; abscissae: time after injection.

As can be seen the total amount of Triton closely parallels the yield of protein. This is stressed by the fact that both the total tritosomal radioactivity and the total protein content of tritosomes were determined in independent experiments. A retardation of the formation of light tritosomes between 24 and 48 hours is apparent. A more complicated picture is seen in the heavy tritosomal fraction. The storage of [$^3$H]Triton in the heavy tritosomes rises continuously up to 48 h and then declines, whereas the yield of protein falls sharply after 12 h and parallels the radioactivity after 24 h.

*Correlation of [$^3$H]Triton to marker enzymes*

In order to study the intracellular localization of [$^3$H]Triton before its appearance in the tritosomes at the beginning of the Triton uptake (1—2 h after injection) density gradient centrifugation studies with cell fractions were performed at the beginning of the Triton uptake (Fig. 6a and 6b).
The 750 × $g$ sediment routinely fractionated into plasma membranes and a nuclear fraction, showed significant radioactivity only in the plasma membrane. The plasma membrane was analysed further by equilibrium density gradient centrifugation on a linear sucrose gradient (0.8—1.6M, 280000 × $g$, 14 h). The radioactivity was closely associated with the plasma membrane. The possibility of lysosomal contamination was excluded since the acid phosphatase proved to be L-(+)-tartrate-insensitive[4].
The microsomal fraction was not further separated, because Triton did not accumulate in this fraction. The 100000 × $g$ supernatant contained no sedimentable radioactivity (100000 × $g$, 14 h).
Gradient centrifugation of the 6500 × $g$ sediment (Fig. 9A) fraction exhibited radioactivity only in correlation to lysosomal acid phosphatase as shown by density gradient centrifugation (1.1—1.9M sucrose, 14 h, 100000 × $g$). The 20000 × $g$ sediment was layered on a continuous density gradient (1.1—1.9M sucrose, 100000 × $g$, 14 h). Fig. 6b shows that the $^3$H-distribution two hours after [$^3$H]Triton injection is closely correlated to the acid phosphatase activity concentrated at a density of $d = 1.18$ corresponding to the usual density of lysosomal acid phosphatase. A minor peak appears at a density of 1.15 representing a population of lysosomes the density of which is changed by early Triton uptake.

*Influence of Triton on the distribution of marker enzymes in the cell fractions*

Glucose-6-phosphatase, 5′-nucleotidase and glucose-6-phosphate dehydrogenase were chosen as marker enzymes for microsomes, plasma membranes and the cell sap respectively. The specific activities of these enzymes remained constant under the influence of Triton up to 96 h in all cell fractions prepared. The recovery of total activity of each

[4] M. W. Neil and M. W. Horner, Biochem. J. **84**, 32P [1962].

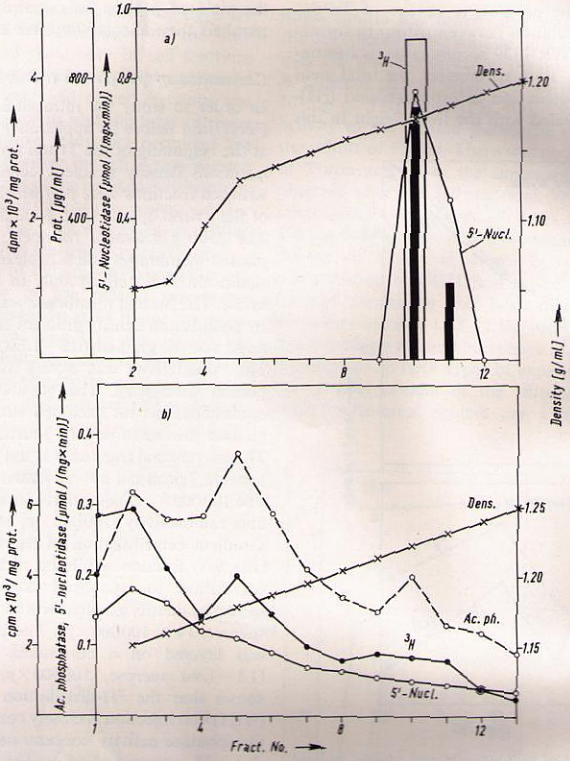


Fig. 6. Correlation of [³H]Triton to marker enzymes studied by linear density gradient centrifugation.
a) Isolated plasma membranes, 1 h after application of 350 mg Triton WR-1339, total radioactivity: $2.88 \times 10^8$ dpm; density gradient: 0.8—1.6M sucrose, centrifugation 14 h at $280000 \times g$.
black columns: µg protein/ml; ×——×: density (0°C); ○——○: 5'-nucleotidase (U/mg protein); empty column: ³H dpm/mg protein.
(Fraction 10 contained insufficient amounts of protein for reliable radioactivity counting.)
b) $20000 \times g$ sediment (Fig. 9a) layered on a 1.2—1.9M linear sucrose gradient, 2 h after [³H]Triton WR-1339 application (dose as in a), 14 h centrifugation at $100000 \times g$.
○---○: acid phosphatase (U/mg protein); ○——○: 5'-nucleotidase (U/mg protein); •——•: ³H dpm/mg of protein; ×——×: density (0°C).

enzyme was found to be in the range of 85—100% of the total activity found in the homogenate. Only the lysosomal marker enzymes, β-glucuronidase and L-(+)-tartrate-sensitive acid phosphatase[4], showed distinct alterations under the action of Triton in the lysosomal fractions and the supernatant (see Fig. 7a and b).

The formation of light tritosomes can be followed by the specific activities of acid phosphatase and β-glucuronidase. This corresponds well to the

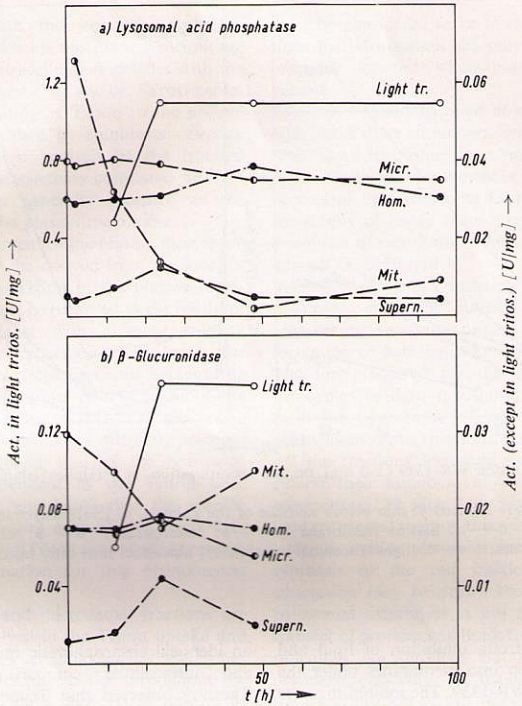


Fig. 7. Distribution of acid phosphatase (L-(+)-tartrate sensitive) and β-glucuronidase in cell fractions up to 96 h after injection of Triton WR-1339 (350 mg).

a) Acid phosphatase; b) β-glucuronidase.

○——○: light tritosomes; ●–·–·●: homogenate; ◐– – –◐: microsomes; ◑– – –◑: mitochondria; ●– – –●: cell sap (supernatant); abscissae: time after injection.

accumulation of [$^3$H]Triton in this tritosomal fraction as shown in Fig. 4a. The concomitant decrease of the lysosomal marker enzyme in the mitochondrial fraction indicates the disappearance of lysosomes from this fraction. An increase of the lysosomal marker enzymes together with the increase of [$^3$H]Triton in the supernatant was observed at 24 h. (Fig. 4a). Again this phenomenon is absent after the application of a small dose of Triton. The nearly constant specific activity of the homogenate and of the microsomes does not suggest a significant net increase in the synthesis of these enzymes.

*Membrane synthesis after Triton application*

[$^3$H]Phenylalanine and [$^{14}$C]choline were injected simultaneously into the portal vein in order to study the overall synthesis of membranes in the liver cell as a function of the time after Triton application. The livers were fractionated 45 min after the precursor injection and the incorporation into cellular fractions was determined.

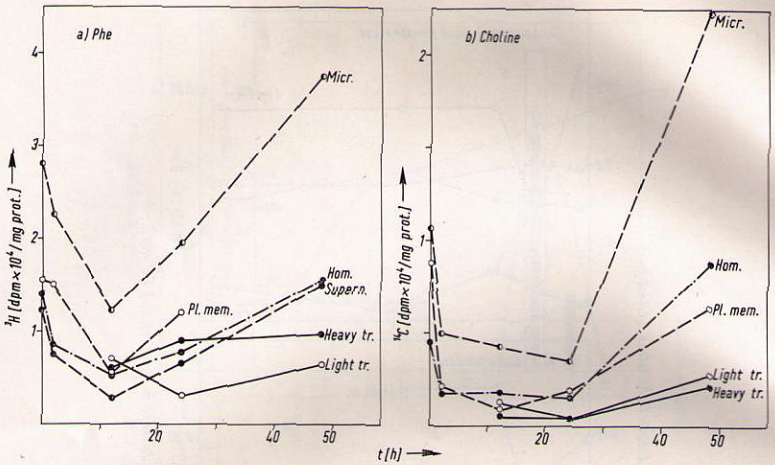


Fig. 8. Influence of Triton WR-1339 (350 mg) on the incorporation of [$^3$H]phenylalanine and [$^{14}$C]choline into cell fractions.
Labelled precursors were injected 45 min before sacrifice of the animals. a) [$^3$H]Phenylalanine; b) [$^{14}$C]choline. o---o: Microsomes; o---o: plasma membrane; •-·-•: homogenate; •---•: cell sap (supernatant); •——•: heavy tritosomes; o——o: light tritosomal membranes; abscissae: time after injection.

Fig. 8 shows the strong inhibition of lipid and protein incorporation into membranes under the influence of Triton WR-1339. The inhibition starts within the first two hours and the synthesis returns to normal between 24 and 48 h after the Triton application. The depression of lipid synthesis is nearly constant up to 24 h whereas protein synthesis shows a sharp minimum at about 12 h. It is noteworthy that the minimum of the precursor incorporation into the light tritosomal membranes lies 12 h later than that of the other cell fractions.

## Discussion

The results of paper electrophoresis and polyacrylamide disc electrophoresis suggest that the labelled Triton WR-1339 is transported bound to a serum protein, an assumption which was also supported by *in vitro* incubation with native rat serum.
Furthermore electrophoresis of mixed samples of various compartments (serum, cell sap, tritosomes) indicates that the [$^3$H]Triton protein complex has an identical electrophoretic mobility in all extra- and intracellular compartments investigated. TROUET[5] observed that Triton forms a complex with α-globulin which he found within the tritosomes. The electrophoretic mobility in our experiments, however, shows an even lower mobility than that of α-globulins.
The barrier between the blood plasma and the inner compartments of the cell is the plasma membrane. Its involvement in transport processes of macromolecules has been studied extensively by light and electron microscopy (for references see STRAUS[6,7], DAEMS *et al.*[8]). In this report its participation in the transport of Triton is shown by

[5] A. TROUET, Arch. int. Physiol. Biochim. **72**, 698 [1964].
[6] W. STRAUS, J. Cell Biol. **20**, 497 [1964].
[7] W. STRAUS, in D. B. RODYN: Enzyme Cytology, p. 239, Acad. Press London 1967.
[8] W. Th. DAEMS, E. WISSE and P. BREDEROO, in J. T. DINGLE and H. B. FELL: Lysosomes in Biology and Pathology, Vol. I, p. 64, Amsterdam, London, North Holland, Publ. Comp. 1969.

results obtained with the isolated membrane. Firstly, Triton uptake by the plasma membrane exceeds that of all intracellular organelles with the exception of tritosomes (Fig. 4a, b). Experimental evidence for the binding of Triton to the plasma membrane was furnished by equilibrium density gradient centrifugation analysis of the isolated membrane. Its $^3$H-radioactivity correlated strongly with 5'-nucleotidase, generally accepted as the marker enzyme of the plasma membrane.

Secondly the involvement of the plasma membrane in the transport may be derived from the kinetics of the Triton concentration in the plasma membrane. The kinetics may be regarded as the resultant of uptake and release with different velocity constants for these processes. Considering the plasma membrane as a compartment between the blood plasma and the inner compartment of the cell the attainment of the maximum of the Triton concentration in this membrane distinctly precedes that of the homogenate and of the lysosomal compartment, independent of the Triton dose administered. It is remarkable that the specific activity remains constant even after a thirty-five-fold dilution of the [$^3$H]Triton is applied. At present a reasonable explanation for this phenomenon cannot be offered.

The mitochondrial and microsomal fractions are apparently not involved in the Triton uptake and distribution processes as indicated by their constantly low specific radioactivities throughout the observation period after a high Triton dose. This is in contrast to observations of Wattiaux *et al.*[3], who reported a high labelling of the microsomal fraction using $^{125}$J-Triton WR-1339.

A low dose of Triton is not able to alter the density of lysosomes and therefore most of the lysosomal-bound Triton remains in the mitochondrial fraction. The kinetics of the radioactivity of the mitochondrial fraction containing the bulk of lysosomes must therefore be regarded as the Triton uptake by the lysosomal system of the cell. It should be stressed that the formation of light tritosomes is strictly dependent on the application of the high dose since light tritosomes do not appear after application of a small Triton dose.

The kinetics of the biogenesis of the light tritosomes can be followed (see Fig. 5a and b) by measuring the total yield of light tritosomal protein and $^3$H-radioactivity. It demonstrates the relationship of the formation of light tritosomes and Triton accumulation. The origin of these light tritosomes must be considered to be in the heavy tritosomes since light tritosomes are only formed to an appreciable amount when heavy tritosomes are present.

This can be demonstrated at zero time and 24 h after the Triton administration (Fig. 5b). At zero time heavy tritosomes have not yet been formed, between 12 and 24 h they have been utilized in the process of light tritosome formation. In addition, the supply of heavy tritosomes is stopped by the inhibition of membrane synthesis during this time interval (Fig. 8a and b).

In the final phase of Triton uptake heavy tritosomes re-appear, since the activity of the membrane synthesizing apparatus returns to normal and the formation of light tritosomes starts again.

The time required for the transition of heavy tritosomes to light tritosomes may be estimated from the appearance of the minimum of [$^3$H]-phenylalanine incorporation into the cell fractions. All the fractions including the heavy tritosomal exhibit their minimum in [$^3$H]phenylalanine incorporation 12 h after Triton injection. Only in the light tritosomal fraction this minimum appears 12 h later. A similar picture is seen in the lipid biosynthesis of the cell fractions. Therefore the conclusion may be drawn that initially the light tritosomal membrane is not formed by a direct transfer of proteins and lipids from the endoplasmic reticulum but develops out of an intermediate organelle. Secondly, the maximum time required for the transition of this intermediate organelle is estimated to be 12 h. This is concluded from the earliest appearance of light Triton-filled lysosomes 12 h after Triton injection, (Fig. 4a) and the depressed formation of the light tritosomes 12 h after maximum inhibition of the protein synthesis (Fig. 5a and b). The intermediate organelles are assumed to be the heavy tritosomes which gradually develop during a period of several hours to light tritosomes.

At least two possible ways of entrance of Triton into the heavy lysosomes must be discussed. The first is a direct transfer from the plasma membrane *via* pinocytotic vesicles. Such vesicles could not be detected in density gradient centrifugation experiments in the early phase of Triton uptake. The only appreciable accumulation of particle bound Triton was found in association with typical lysosomal acid phosphatase (Fig. 6b). The second way could be a shifting concentration of Triton from the soluble compartment of the cell into the

tritosomes since the disappearance of Triton from the soluble fraction and its increase in the heavy tritosomes can be correlated and explained as an intracellular displacement from the soluble fraction into the heavy tritosomes. This is an expression of a disordered cellular metabolism[9] and indeed the severe derangement of protein and lipid synthesis support this point. (Fig. 8a und b).

The maximum concentration of Triton in the cell sap is accompanied by a release of lysosomal enzymes into the soluble compartment as indicated by the increase of the specific activities of β-glucuronidase and lysosomal acid phosphatase (Fig. 7a, b). An analogous example has been described for autophagocytosis caused by glucagon application[10,11]. In this connection the question has to be raised whether the primary event which leads to these serious metabolic perturbations is due to Triton permeation through the plasma membrane and the direct contact of the detergent with the synthetic apparatus or whether a rupture of lysosomes by Triton overloading with concomitant enzyme release starts the metabolic disturbances. An exact answer cannot be given because of the uncertainty concerning the nature of the Triton transport through the surface membrane as mentioned above. In the case of the high Triton concentration the possibility of a direct permeation cannot be ruled out. Two observations may support this mode of entrance. Firstly, the thirty-fold dilution of [$^3$H]Triton with inactive Triton results in a remarkable decrease of the specific radioactivity in the blood serum (Fig. 2) which means that its binding capacity is overloaded. Since it has been shown that Triton is only found in a bound form the excess of Triton not bound to serum is eliminated from the circulation. This loss of unbound Triton might be explained by a rapid penetration through cellular membranes due to its detergent nature. Secondly, the time sequence of events shows that a significant lysosomal rupture or leakage (12—24 h) occurs after the initiation of serious cellular damage. Since the inhibition of membrane synthesis by Triton preceeds the release of lysosomal enzymes it may be concluded that Triton initiates the cell injury.

Since, however, our results fail to give definite experimental evidence for this explanation, the possibility of a vesicle associated transport into the cell still remains. An approach to the problem of [$^3$H]Triton uptake by the liver cell by electronmicroscopic autoradiography is in progress.

On the basis of these results pinocytosis or a permeation process (transport?) with subsequent lysosomal concentration analogous to the intracellular autophagocytosis may be discussed. The chemical and enzymatic comparison of the plasma and lysosomal membrane[1,2] do not lend experimental support to the pinocytosis proposal. On the other hand the kinetic data obtained by the small Triton dose are not at variance with the concept of pinocytosis. So far the experiments do not give conclusive evidence for a solely pinocytotic uptake of Triton WR-1339.

The complexity of the formation of the tritosomal membrane also becomes apparent when the phospholipid composition of the tritosomal membranes isolated 48 and 96 h after the injection is compared[12]. An increase in the content of phosphatidylinositol and phosphatidylserine at the expense of phosphatidylcholine and sphingomyelin is found in the 96 h lysosomal membranes. This is in agreement with the demonstration of an increase of phosphatidylinositol and phosphatidylserine synthesis in analogous processes such as phagocytosis[13] and secretion[14]. When compared with the phospholipid composition of the plasma membrane[1] this change in the composition of the lipids refers to a decreasing contribution of the plasma membrane to the formation of the secondary lysosomal membrane.

In summary our results indicate that the plasma membrane is involved in the transport of Triton WR-1339. The nature of this process, however, cannot yet be exactly described. The predominant way of Triton entrance cannot be quantitated because the isolation of the intracellular vesicles produced either by pinocytosis or autophagocytosis was not possible. Our present data can be inter-

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preted as a combination of pinocytosis and intracellular lysosomal concentration of Triton WR-1339.

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## Material and Methods

*Experimental materials and reagents*

Triton WR-1339 was purchased from Serva, Heidelberg. $^3$H-Labelled Triton WR-1339 was kindly prepared by Prof. Dr. G. Stöcklin (Kernforschungsanlage Jülich, Germany) by the Wilzbach technique (spec. radioactivity: $2.88 \times 10^7$ dpm/mg). *p*-Nitrophenylphosphate and *p*-nitrophenyl-β-D-glucopyranosiduronic acid were obtained from Merck, Darmstadt, glucose 6-phosphate, 5′-AMP and NADP$^{\oplus}$ from Boehringer Mannheim GmbH. [*methyl*-$^{14}$C]Choline chloride (spec. activity: 37.6 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. [$^3$H]-Phenylalanine (spec. activity: 33 Ci/mmol) was kindly provided by Prof. Dr. K. Hempel, University of Würzburg. 36.4 μCi of [$^3$H]phenylalanine and 7.7 μCi of [$^{14}$C]choline dissolved in 0.25 m*l* of 0.9% NaCl solution were injected into the portal vein of rats weighing about 200 g 45 min before sacrifice.

*Preparation of subcellular fractions*

The homogenization procedures, the isolation of plasma membranes, Triton WR-1339 filled lysosomes ("tritosomes") and tritosomal membranes has been described previously[1]. In this report two cell fractionation procedures have been applied. Procedure A was used when plasma membranes, mitochondria, a mitochondrial-lysosomal fraction, microsomes and the final supernatant (cell sap) were isolated 0—2 h after Triton injection (Fig. 9 A). In procedure B plasma membranes, mitochondria, heavy tritosomes (density 1.13—1.18), light tritosomes (density 1.12), a 12000—20000 × *g* (10 min) sediment, microsomes and the cell sap were isolated up to 96 h (Fig. 9 B). The fraction of density 1.13—1.18 is called heavy tritosomes because of its increase in latent lysosomal acid phosphatase and the accumulation of Triton.

*Analytical procedures*

Protein was determined by the Lowry method[15], 5′-Nucleotidase, glucose-6-phosphatase, cytochrome *c* oxidase and acid phosphatase determinations were described earlier[1].

β-Glucuronidase was determined according to Gianetto and de Duve[16] and glucose-6-phosphate dehydrogenase according to Löhr and Waller[17].

Polyacrylamide gel disc electrophoresis was performed in 7.5% gel, pH 8.3 (Maurer[18], system Nr. 1a). For the measurement of radioactivity the gels were frozen and cut into 1 mm pieces with a transversal gel slicer. The slices were dissolved in 0.25 m*l* of 30% $H_2O_2$ at 50°C (5 h). After the addition of 1 m*l* of Soluene 100 (Packard)/isopropanol 1:1 the samples were counted after mixing with 10 m*l* of a scintillator containing 5 g of PPO (2,3-diphenyloxazole) and 0.3 g of POPOP [1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene] in 1 *l* of toluene. Cellulose acetate electrophoresis was carried out with 0.1 M veronal* acetate buffer, pH 9.0 using 6 μg—5 mg of protein depending on the specific radioactivity. The strips were cut into 0.5 cm pieces, dissolved and counted in Bray's solution[19]. In mixed samples equal amounts of total radioactivity were applied. Binding of [$^3$H]Triton to serum protein was studied after incubation of 2 μg [$^3$H]Triton with 50 μ*l* of native rat serum for 60 min at 37°C. Light tritosomes were briefly sonicated before application to electrophoresis.

Radioactivity of [$^3$H]Triton in cell fractions was determined after solubilization of 50 μ*l* portions with Soluene 100 (Packard) in the above-mentioned toluene scintillator. Counting efficiencies were determined by the external standard method in a Packard Tri-Carb Scintillation Counter (model 3380/544). [$^3$H]Phenylalanine and [$^{14}$C]choline incorporation was measured in 5 times washed 5% trichloroacetic acid precipitates solubilized with Soluene 100. In preliminary experiments the lipids of cell fractions were extracted and [$^{14}$C]choline was shown to be transferred during the incorporation period only into phosphatidylcholine as proved by radio-thin-layer chromatography (silica gel H, solvent system chloroform/methanol/water 65:25:4).

* The name recommended bei WHO is "barbital".

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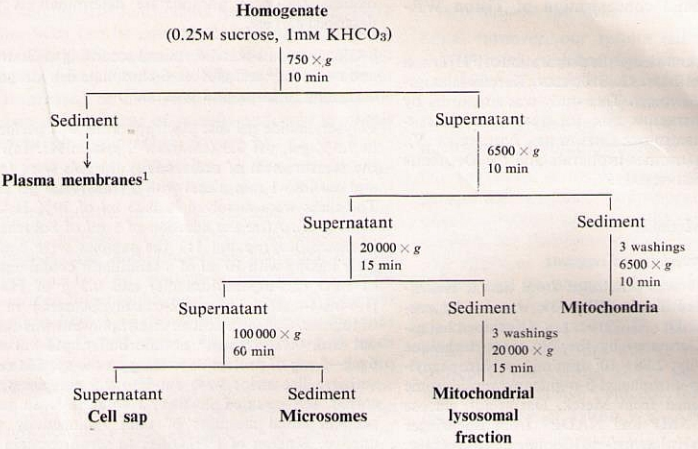
a) Procedure A:




b) Procedure B:

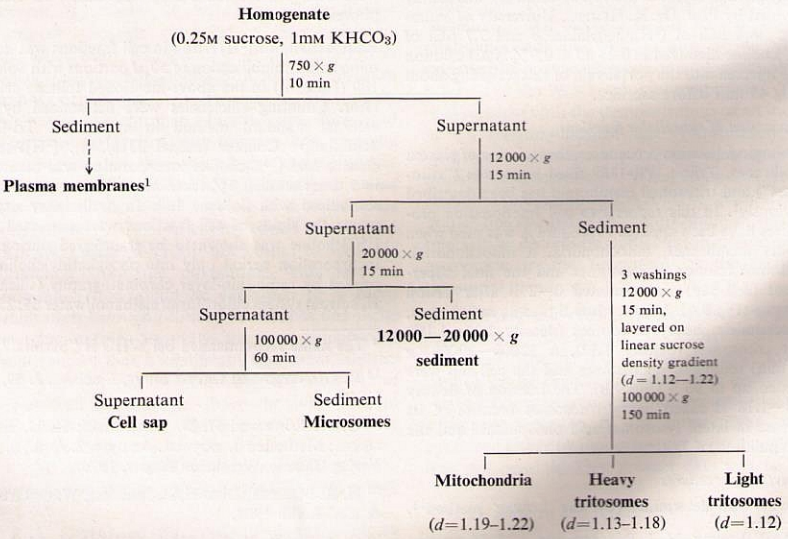



Fig. 9. Cell fractionation procedures. Procedure A used 0—2 h after Triton WR-1339 injection; procedure B: used 2—96 h after Triton WR-1339 injection.