The Fate of ‘Thorotrast’ (Thorium Dioxide) injected into the Dorsal Lymph Sac of the Frog, *Rana temporaria*

By G. E. H. FOXON and K. E. K. ROWSON

(From the Departments of Biology and Pathology, Guy’s Hospital Medical School, London, S.E. 1)

With 5 plates (figs. 1–5)

SUMMARY

Thorotrast (a colloidal suspension of thorium dioxide) injected into the dorsal lymph sac of the common frog, *Rana temporaria*, can be shown by radiological methods to pass through the anterior lymph hearts and so into the blood system. From the blood the thorotrast is removed, as in mammals, by cells of the reticulo-endothelial system and by macrophages, the sites of greatest activity being the liver, spleen, and bone-marrow. Whereas in mammals almost all uptake of such colloidal substances takes place in these organs, in the frog there is considerable macrophage activity elsewhere, notably in the submucosa of the alimentary canal. Our results suggest that macrophages which ingest thorium particles in the wall of the alimentary canal migrate through the blood system to the liver where the thorium is ultimately deposited. This may be correlated with the possession by the frog of a macrophage and reticulo-endothelial system less highly organized than that possessed by mammals.

INTRODUCTION

WHEN it is desired to ascertain the reactions of frogs to various drugs and other materials, the dorsal lymph sac is frequently utilized as a site of injection. The use of the dorsal lymph sac for this purpose is favourable on account of the very rapid lymph circulation, which has been investigated in some detail in *Rana pipiens* by Conklin (1930), who found that this lymph circulation dealt with amounts of fluid which, when compared with the body weight of the frog and the total blood-volume, were indeed considerable.

It occurred to us that this circulation of lymph might be made visible by the injection into the dorsal lymph sac of the contrast medium ‘thorotrast’ (a colloidal suspension miscible with blood, containing 24–26 per cent. of thorium dioxide by volume), followed by serial radiographs. As our results show, this has been possible and the movement of the thorotrast from the lymph sac to the blood-stream has been demonstrated radiologically. Radt (1930) and Volicer (1931) described the use of thorotrast in hepato-lienography, i.e. the radiological investigation of the liver and spleen. As these authors pointed out, the principle of this technique is that colloidal particles injected into the blood-stream are removed by phagocytic activities of the reticulo-endothelial system and that if such colloidal particles are radio-opaque they produce substantial shadows on an X-ray plate. Such shadows not only show the extent of the organ in which the substance has accumulated but also give information as to the activity of the tissues responsible for its uptake. Although on its introduction this technique of hepato-lienography

was claimed to be harmless to the patient, much controversy has developed over the possible effects of the prolonged storage in the human body of a substance which is radio-active. For, once taken up by the body, there is never any considerable elimination of the thorotrast.

Research on the uptake and fate of thorotrast appears to have been confined to mammals including dogs, rats, mice, guinea-pigs, and rabbits (Irwin, 1932; Tripoli, 1934). There is also information as to the fate of the thorotrast in man after a considerable number of years (see Cassel, Ruffin, Reeves, and Stoddard, 1951). As the result of our injections of thorotrast into the dorsal lymph sac of frogs, we have been able to observe not only its transfer to the blood but its removal from the blood to the liver and spleen. This process of removal seems to us not altogether simple, and we have come upon features of the process which appear to merit description as illustrating the properties of the reticulo-endothelial system.

EXPERIMENTS

Procedure

Specimens of the common frog, *Rana temporaria*, obtained from dealers were used in these experiments. The majority weighed about 20 g, but a few were somewhat heavier. They were kept at room temperature throughout the experiments. The thorotrast was injected with fully aseptic precautions into the dorsal lymph sac. The amount varied. In experiments designed to trace the path of the thorotrast from the lymph sac into the circulation 0.5 c.c. was used, but in the majority of experiments to investigate the ultimate fate of the thorotrast the quantity was reduced to 0.1 c.c. Even this is greatly in excess of the dose of 0.8 c.c. per kilogram of body weight suggested for man and other mammals by Tripoli (1934).

The frogs were radiographed at various intervals after injection. The radiographic factors used were standardized at 100 mA 35 kv 0.4 seconds at a focus-film distance of 100 cm. The exposures were made on envelope-packed ‘Ilfex’ X-ray film (for use without intensifying screens) and the development time was 4 minutes at about 24° C.

The frogs were allowed to survive until such time as it was desired to compare the radiographs with sections of the various organs. During this time the frogs were fed regularly on suitable material such as earthworms.

When the frogs were killed, the organs to be investigated were fixed in Zenker-formaldehyde solution, and after appropriate treatment the resulting sections were stained in Ehrlich’s haematoxylin and counterstained in eosin.

RESULTS

The lymphatic pathway

To show what happens when thorotrast is injected into the dorsal lymph sac of a frog a rather heavy injection (0.5 c.c.) was used. The results of such an experiment are shown in fig. 1.
FIG. 1

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Dorsal Lymph Sac of Rana

The normal frog before injection is shown in fig. 1, A and the state immediately after injection in B. Although the injection was made well anterior to the sacral prominence the thorotrast, presumably because it is heavy, has sunk to the lower parts of the sac, and already, in the few seconds that have elapsed between injection and the making of the X-ray, two streams of thorotrast have begun to move forwards along the margins of the dorsal lymph sac (a in B). In C (15 minutes after injection) a considerable forward movement of the thorotrast has taken place, some of it no doubt due to movements of the frog, and shadows have appeared (b) in the region of the anterior venae cavae, to which the anterior pair of lymph hearts are connected by the vertebral veins. In D (45 minutes after injection) the heart is giving a pronounced shadow (c) as are the vessels in the lungs (d). In addition there are blood-vessels clearly shown in the right leg. E (4 hours after injection) shows that there is little, if any, thorotrast left in the lymph sac. In the region of the heart and sternum (e in E) a dense shadow is to be seen; the lung fields are well filled and the blood-vessels of the legs are very conspicuous. Shadows which have begun to appear may be cast by the spleen and portions of the liver, but this is uncertain.

F shows the frog 24 hours after injection. The blood system is still visible in the fore limbs and more clearly in the hind limbs. The liver has started to cast its unmistakable shadows (f) and there are other shadows in the region of the intestine.

In G (48 hours after injection) the blood system is still marked and the liver is becoming well defined (g).

Finally, H shows the frog 8 days after the injection. The thorotrast has disappeared from the circulation. Dense shadows are cast by the liver and spleen (h) and there is also an accumulation of radio-opaque material in the intestine (j). Although this frog was kept for another 7 days there was no significant change in the shadows. When the frog was killed the shadow in the

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FIG. 1 (plate). A, radiograph of normal frog. B-H, radiographs of the same frog at various times after injection of 0·5 c.c. thorotrast into the dorsal lymph sac. B, immediately after injection. C, 15 minutes after injection. D, 45 minutes after injection. E, 4 hours after injection. F, 24 hours after injection. G, 48 hours after injection. H, 8 days after injection. a, thorotrast passing forwards along the lateral walls of the dorsal lymph sac. b, shadows caused by thorotrast in the anterior venae cavae. c, shadow of heart. d, shadows of vessels of right lung. e, region of heart and sternum. f, shadow of right lobe of liver. g, shadow of right lobe of liver, more intense. h, shadow of spleen. j, shadow caused by mass of radio-opaque material in intestine.

(For full description see text.)
Foxon and Rowson—'Thorotrast' injected into the intestine was found to be caused by a large brown mass, presumably thorotrast, which had obtained access to the intestine. This is not to be regarded as normal. An apparently similar frog injected with the same quantity of thorotrast at the same time did not show any shadow in the lumen of the alimentary canal during a similar period. This result was probably caused by the injection of too large a quantity of thorotrast to be dealt with by the normal mechanism, and so in subsequent experiments the amount injected was considerably reduced, and this circumstance was not met with again. (This matter will be commented upon again later.)

Thus thorotrast injected into the dorsal lymph sac is rapidly removed by the action of the anterior pair of lymph hearts, which, by their contractions, pass the lymph into the vertebral vein on each side of the body and so through the anterior venae cavae into the heart. We were unable to detect in the radiographs the return of any of the thorotrast from the lymph sac by way of the posterior lymph hearts and iliac veins.

The removal of the thorotrast from the blood-stream

In the account just given of the lymphatic pathway it is to be noted that the events following injection of the thorotrast into the dorsal lymph sac are not clearly separable in time but merge into each other; thus the shadows of the liver and spleen begin to become apparent while thorotrast is still in the blood-stream. The process of removal from the blood cannot be separated off, for presumably it begins as soon as thorotrast enters the blood but is not detectable radiologically until it has been taking place for some time.

For the investigation of this process it was found desirable to make a smaller injection than 0.5 c.c., and in an injection with 0.1 c.c. of thorotrast the dilution is such that the blood-vessels show only faintly. The vessels of the legs can be discerned in a radiograph taken 3 hours 45 minutes after injection. A radio-
Fig. 2
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graph taken 24 hours after injection shows the shadow of the liver, and a slightly later radiograph (3 hours later still) is reproduced in fig. 2, A. Fig. 2, B shows a radiograph made 2 days after injection; C, 3 days after, and D, 16 days after injection. The main point of interest is that during this time the liver shadow becomes distinctly more intense. A point we noted was that shadows in the area marked \( x \) in A-D showed some variation, and the explanation that occurred to us was that thorotrast which had been removed in some way from the blood circulation was being transferred to the liver through the portal system. Thus it might be that the final site of the thorotrast was not necessarily that at which it left the blood-stream.

Similar changes in the distribution of thorotrast after its disappearance from the blood-stream were found in other experiments. Fig. 2, E-G shows a frog injected with \( 0.1 \) c.c. thorotrast 4, 8, and 36 days after injection. There is considerable difference between these radiographs in the distribution of the thorotrast.

There are various shadows in the region of the intestine which have been shown, by removing the alimentary canal and radiographing it outside the body, to be caused by thorotrast in the spleen and also in neighbouring tissues, including the wall of the alimentary canal itself. This is well shown in fig. 4, E and F.

The distribution of thorotrast between liver and spleen at an early stage in its removal from the blood system is by no means constant. In fig. 2, A (29 hours after injection) there is a well-defined liver shadow. In another frog similarly injected (fig. 2, H, 28 hours after injection) the spleen shows up well and the liver hardly at all. (The liver shows up slightly in the original X-ray, but in printing to obtain contrast between the spleen and the vertebral column the liver ceases to be apparent.)

There is therefore some degree of variation in what may take place when the thorotrast is removed from the blood. Such variation may be correlated with variation in the activity of the cells of the reticulo-endothelial system. In rats the activities of these cells have been shown by Gordon and Katsh (1952) to be associated with the nutritional state of the animal and the activity of its adrenal glands. Some of the variation in the uptake of the thorotrast which we have described may have been due to such causes.

Some of the movements of the thorotrast after it has left the blood are of considerable duration. For example, in fig. 2, I-L are shown the conditions in the same frog 2, 7, 24, and 133 days after injection respectively. Accumulation of thorotrast in the liver and the gradual diminution in the size of the spleen is readily seen.

These relatively slow movements of thorotrast led us to inquire into the actual method by which it leaves the blood and accumulates in the liver and spleen. In order to do this, sections were cut of organs from injected animals and an attempt made to correlate the micro-anatomy with the radiological anatomy.

The particles of thorotrast as injected are not visible under the microscope,
but in cells it appears as highly refractile particles. How this comes about is not at present clear. At some stage flocculation of the colloidal particles must take place. According to Irwin (1932) flocculation in his rabbits took place after 5 minutes.

The course of events seen in the liver will now be described.

Fig. 3, A shows the section of normal liver of frog; the characteristic cells containing melanin are shown at m. Fig. 3, B shows that by the time 24 hours have elapsed after injection thorotrast is found in the liver sinusoids. Some of this is clearly inside cells. After 48 hours cells containing thorotrast are found in practically all the sinusoids, and after 14 days even larger aggregations of these cells are seen. At 52 days (fig. 3, C) the cells in the sinusoids have become more numerous, but at 133 days (fig. 3, D), while there are still some aggregates of large cells in the sinusoids, thorotrast is clearly visible in the liver-cells themselves. Thorotrast is also absorbed by the cells containing melanin. (Tripoli (1934) using smaller doses found that in the mammals on which he worked, although there might be an occasional thorium particle in the liver-cells after 1 month, later no such particles were to be seen; all being within the reticulo-endothelial cells.)

Some of the details of this sequence of events is shown after high magnification in fig. 3, E (after 52 days) and F (after 133 days). After 48 hours the thorotrast is mainly confined to the sinusoids; the 52-day (E) specimen showed some tendency for the thorotrast to be accumulated in the liver-cells away from the sinusoids. At 133 days (F) many of the sinusoids were clear, but thorotrast was visible in the cells where in some cases it appeared to surround the nuclei.

We interpret these results as follows: in the liver thorotrast is first picked up by reticulo-endothelial cells and macrophages which appear to lie free in the sinusoids, but which may be reticulo-endothelial cells which first enlarge and then perhaps become free in the sinusoids; that these cells then tend to coalesce, or at least aggregate, and that then some process of transference of the thorotrast from these cells to the parenchyma cells takes place. This results in a diminution after some weeks in the number of cells containing thorotrast; these cells almost block the sinusoids. As will be seen later, this apparent sequence of events is not the same as that described for the rabbit by Irwin (1932).

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**Fig. 3** (plate).

Photomicrographs of sections of liver of (A) normal and (B-F) experimental frogs injected with 0.1 c.c. thorotrast. A to D magnified as fig. 5, C.

B, 24 hours after injection.
C, 52 days after injection.
D, 133 days after injection.
E, 52 days after injection
F, 133 days after injection
magnified as fig. 5, F.

m, cells containing thorium particles, in sinusoids.

m, cells containing melanin.
t, thorium particles in liver-cells.
FIG. 3

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FIG. 4

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In the spleen the succession of events is shown in fig. 4, A–D. A shows normal frog spleen. B (after 72 hours) shows the accumulation of thorotrast in the spleen, some particles definitely in cells but some only doubtfully so. C (after 42 days) shows a tendency to form larger aggregations, and D (133 days after injection) shows some very large masses of thorotrast indeed. The coming together of phagocytic cells containing thorotrast to form composite structures in the spleen of the rabbit has been described by Irwin (1932). It will be remembered, however, that when a spleen was observed at intervals over a considerable period it showed a diminution in size (fig. 2, I–L).

The combination of these results with those obtained from radiography suggests that there might be a gradual transference of thorotrast from the spleen to the liver, and previous radiographs, as has already been remarked, suggest that there might be other movements going on, particularly in the early stages, as transient shadows had appeared and disappeared; this suggested that thorotrast might be present in other parts of the body shortly after injection, particularly in the walls of some parts of the alimentary canal.

Some heavier injections show the accumulation of thorotrast in the walls of the alimentary canal. For example, in fig. 4, E is shown the alimentary canal of a frog injected 20 days previously with 0.4 c.c. thorotrast. The accumulation in the liver and spleen is well shown, but there is also much thorotrast in the walls of the stomach. The lines running along the length of this organ were found, on sectioning, to be caused by the deposit of thorotrast in the ridges of the mucosa. The finding of thorotrast in the mucosa is unusual as it is usually confined to the submucosa. However, the injection was a heavy one, and the fact that the thorotrast is in the cells of the surface layer of the stomach suggests an explanation for the condition found in the first experiment described, where thorotrast was apparently found in the lumen of the alimentary canal.

With the injection of 0.1 c.c. thorotrast the shadows in the wall of the alimentary canal are much less distinct than this, but in order to find out whether it was possible to follow the passage of the thorotrast from the wall of the alimentary canal to the liver, a frog, into which 0.1 c.c. of thorotrast had been injected 30 hours previously, was killed and subsequently sections
showed that thorotrast was present (possibly in endothelial cells) in the glomeruli of the kidney (fig. 5, A), in the interstitial tissue of the pancreas (fig. 5, B), and most particularly in the submucosa of the duodenum (fig. 5, C), where it appeared to be clearly in macrophage cells. Fig. 5, D shows the wall and lumen of a vein in the duodenal region, and cells containing thorotrast are seen in the wall of the vessel. Sections of organs of animals similarly treated but which were cut at later times (even 14 days later) do not show the presence of such cells, and this suggests that the cells migrate with the contained thorotrast. It is a well-known fact that the venous drainage of the wall of the alimentary canal favours transport through the portal system to the liver (Wright, 1954, p. 56). With this in mind serial sections were cut through the region of the portal vein of a frog injected 30 hours previously, and in several sections obvious white blood-cells containing thorotrast were found free in the lumen of the vein, and one of these is shown in fig. 5, E and under higher power in fig. 5, F.

Fig. 4, F, which shows the alimentary canal of a frog killed 9 days after injection with 0.5 c.c. thorotrast, shows a marked accumulation in the spleen and also in the surrounding blood-vessels, again suggesting that the thorotrast has been cleared from the wall of the alimentary canal and is being transported through the portal system.

Two other regions which have not so far been mentioned have been examined for the presence of thorotrast.

The first situation is the marrow of the long bones. The presence of thorotrast here is not suggested by the X-rays, but nevertheless smear preparations of marrow from the femur of a frog killed 133 days after injection showed the presence of a considerable quantity included in marrow-cells.

The second situation is the vessels of the lungs. Irwin (1932), working on the rabbit, found cells containing thorotrast in lung tissue 3 months after injection although previously they had not been present. He suggested that cells containing thorotrast wandered out from the liver through the circulation to the lungs and that they passed out into the alveoli and thus the thorotrast was excreted from the body. Such excretion cannot assume large quantities in mammals. In man thorotrast persists in the liver and spleen for considerable periods, presumably for life (see Cassel and others, 1951). We found no such cells in the lungs even in the frog which survived for 133 days.

**Fig. 5 (plate).**
A–D, photomicrographs of sections of organs of a frog injected 30 hours previously with 0.1 c.c. thorotrast. A and B to same scale as C.

A, kidney showing thorium particles in the glomerulus, \(tg\).

B, pancreas showing thorium particles in interstitial cells, \(ti\).

C, duodenum showing cells in submucosa containing thorium, \(tsm\).

D, blood-vessel of portal system showing \(l\), lumen; \(w\), wall, and \(tw\), thorium particles in cells in the wall.

E–F, a cell containing thorium particles in a blood-vessel.

E, a cell \(c\), lying in the lumen of a blood-vessel together with red blood corpuscles, \(rbc\). The frog had been injected 30 hours earlier with 0.1 c.c. thorotrast.

F, the same cell as shown in \(E\) but more highly magnified, showing the thorium granules, \(tg\).
FIG. 5
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DISCUSSION

Our results lead us to the following conclusions: thorotrast injected into the dorsal lymph sac of the frog passes rapidly through the anterior lymph hearts into the blood-stream. From the blood-stream it is progressively removed by the action of cells of the body possessing the properties of macrophages.

These may include certain of the reticulo-endothelial cells of liver and cells of the bone-marrow and also similar cells in other parts of the body, particularly those occurring in the walls of the alimentary canal. These cells are to be regarded as 'resting-wandering' cells or macrophages, and we consider that the results both of our radiographic studies and of the micro-anatomy support this hypothesis.

If this hypothesis is correct, then the rapidity of the removal of the thorotrast from the blood-stream will depend on the number of macrophages which are initially available for removing the thorotrast.

In the first experiment recorded, when 0.5 c.c. of thorotrast was injected, it was visible circulating in the blood up to and including the 2nd day after injection, but it had all disappeared by the 5th day. When 0.1 c.c. was injected, the shadow in the blood-vessels of the legs which was visible after 3 hours 45 minutes had disappeared after 24 hours. This suggests that, when the quantity injected was great, additional macrophage cells were produced to remove the thorotrast and that this took time. Maximow and Bloom (1948, p. 98) state that macrophages in defensive action cannot be sharply separated from lymphoidal cells and that it seems probable that in all defence reactions some new macrophages arise locally from the mitotic division of pre-existing macrophages or by the direct assumption of phagocytic activity by cells having mesenchymal potencies. These authors also point out that, when required to combat general infections or to take up vital dyes, large numbers of free macrophages are mobilized in the liver, bone-marrow, or spleen. Some which pass into venous sinuses are carried by the blood-stream into the right side of the heart and so eventually to the lungs, where most are filtered off in the capillaries, few passing into the general circulation.

Our results are not, in general, out of keeping with this general statement, but it would appear that in the frog the macrophages in the gut wall which contain thorotrast generally migrate into the portal system and that they are transported to the liver where they come to block the sinusoids; here they may be joined by similar cells coming from the spleen. Eventually, we believe, their contents pass into the liver-cells themselves and the macrophages disappear, but as to what is their actual fate we have no evidence.

Irwin (1932), working on the rabbit, interpreted his observations differently. He concluded that the thorotrast was removed from the blood into the parenchyma and Kupffer cells of the liver almost immediately after injection. Later the quantity visible in the parenchyma cells declined while that seen in the Kupffer cells increased; eventually, starting after an interval of 3 months, the Kupffer cells became free in the blood and were passed to the lungs where they
lodged in the fine capillaries and the cells passed through into the alveoli. Here they died and their remains were removed by ciliary action of the bronchi. Hence there was really an excretion of thorotrast. As already remarked, in a frog 133 days after injection no trace of thorotrast could be found in the lungs. Tripoli (1934) also found that in various mammals thorotrast was eliminated from the parenchyma cells of the liver within a comparatively short time (1 month).

A point of interest is the obviously large amount of thorotrast which is removed from the blood by macrophages in the wall of the alimentary canal. This we believe may be correlated with the zoological position of the frog. Jordan (1933) has reviewed the evolution of the spleen and shown that the spleen tissue is embedded in the wall of the intestine in certain fishes. Here it combines erythro- and lymphocytopoietic functions. In higher animals these functions become separated, the marrow of the long bones taking on the former and the spleen the latter. In the Anura, of which the frog is one, the spleen has taken on the lymphocytopoietic function but also forms erythrocytes at certain stages of the life cycle. The group is evolutionarily the lowest in which the bone-marrow has an erythrocytopoietic function. It is known, however, that the lymphocytopoietic function is still retained in part by the alimentary canal in mammals and probably even to a greater degree in frogs, and much of the division of the macrophages appears to take place in this region. It is also true that the macrophages must undertake the ingestion of the thorotrast particles in sluggish parts of the circulation, and it would seem that the capillaries of the wall of the alimentary canal provide a favourable location for this. Also, particles of thorotrast which have passed out of the blood-stream altogether in the connective tissue fluid are engulfed by macrophages in the connective tissue. That thorotrast can occur in cells other than macrophages and parenchyma cells of the liver has already been shown in connexion with the stomach (fig. 4, e). Indeed it is well known that many cells, besides those of the reticulo-endothelial system, take up colloidal particles if a sufficiently large quantity is injected.

The variation that was found in the appearance of liver and spleen in various frogs some 28–30 hours after injection has been noted. This is of particular interest in view of the work of Gaunt and Wright (1940) on rabbits, who found a very constant relationship between the amounts in the three regions, liver, spleen, and bone-marrow. They found about half the thorium could be detected chemically in the liver, one-quarter in the bone-marrow, and one-eighth in the spleen. We have not made any chemical analyses, but it would seem unlikely that such a constant relationship could have been detected. Again, this may be correlated with the lower evolutionary position of the frog, the mechanism responsible for the removal of the thorotrast not having reached such a stable condition as is found in mammals. As already noted, however, it may be correlated with the nutritional condition of the animal at the beginning of the experiment.

In conclusion, we should remark that while the details of our observations
at some points agree with, and at others diverge from those of workers who have studied the reactions of mammalian tissues to the injection of thorotrast, the two points which we consider of most interest in the present study are, first, the actual radiographic demonstration of the movement of the lymph in the lymph spaces of the frog; and second, the evidence which appears to show that cells with phagocytic properties and which engulf thorium dioxide particles migrate about the body, and that these migrations lead ultimately to the liver.

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