

Cytological Studies of the Acinar Cells of the Pancreas of the Mouse

Part I. The Formation of Neutral Red Granules

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With one plate (fig. 1)

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SUMMARY

1. It has been found that during a 12-15 hour period after the subcutaneous injection of mice with a predetermined 'optimum' dose of neutral red, there occurs a definite series of changes in the pancreas acinar cell in which neutral red granules are formed as separate bodies, which subsequently come together as aggregates and then disappear from the cell.
2. The cell is not damaged by the cycle of events just described. The response to the presence of neutral red appears to be physiological, not pathological.
3. Evidence is brought forward showing that neutral red granules and aggregates are not vacuoles that pre-existed in the normal cell and were subsequently stained: they are new formations produced by the presence of neutral red in the cell.
4. Little more than one-quarter of the neutral red injected is excreted in unchanged form.

INTRODUCTION

IN undertaking a cytological study of the exocrine cell of the pancreas of the mouse, the writer had occasion to use neutral red as a vital dye. Neutral red has been much used in attempts to elucidate the true structure of the so-called Golgi apparatus. Its use has given rise to controversy among cytologists. Some have supposed that the dye colours pre-existing vacuoles or 'granules' in the cytoplasm, generally situated in or close to the part of the cell in which the Golgi apparatus is seen in fixed preparations, while others have claimed that the dye does not stain pre-existing objects but *causes* the production of vacuoles or granules as new formations not represented in the

Quarterly Journal Microscopical Science, Vol. 94, part 2, pp. 141-153, June 1953.

normal cell. Different authors, describing the same kind of cell, often give different information as to the number, size, position, and state of separation or aggregation of the neutral red granules.

It occurred to the writer that many of the discrepancies in the accounts given by other investigators might be due to the lack of any systematic attempt to discover the *dosage* of the dye that would give the most valuable information. Those who have studied the pancreas of the mouse have injected widely different amounts. Thus Covell (1928) gave a single injection of 1.5 ml. of a 1 per cent. solution of the dye; Chlopin (1927) gave repeated injections; Beams (1930) gave up to 12 ml. of a saturated solution. Some authors injected the dye subcutaneously, others intraperitoneally, while others again immersed pieces of pancreatic tissue in a solution of the dye. Not much attention has usually been paid to the exact period of time elapsing between injection and microscopical examination.

The writer decided to try to standardize what will here be called an optimum dose, that is to say, a dose that would be sufficient to show neutral red granules clearly, but would not cause gross pathological changes nor prevent the cell from returning in the course of time to a perfectly normal condition. He also decided to make his observations at precisely measured times after injection, so as to find whether there was any regularly occurring cycle in the appearance, growth, and disappearance of the granules.

MATERIAL AND METHODS

Adult male mice weighing 25 to 35 gm. were used exclusively in this study. Approximately 200 mice from several different lots were studied.

Neutral red chloride, the only form of neutral red tried, was obtained from four different makers. They were: British Drug Houses, Ltd. (batch numbers 673505/510228 and 672311/510615); Hopkin and Williams Ltd. (batch number 31517); G. T. Gurr (batch number 04955); and Edward Gurr Ltd. (batch number 352/1). The results with all of these were essentially the same.

The fluid in which the neutral red powder was dissolved for injection did not appear to influence the results materially. Both normal saline and distilled water were used. The latter, however, on the whole gave the best results.

Mice were always taken from a feeding stock cage, injected subcutaneously on the sides or back, and killed by a blow on the head. The site of injection chosen would seem to be of some importance, for when injection over the abdomen is attempted the abdominal wall is easily punctured, and one cannot always be certain that the injection has not been given intraperitoneally. It may be said in passing that the practice of introducing the neutral red subcutaneously obviates the criticism that intraperitoneal injection of distilled water causes abnormal changes in the pancreas (Gatenby, 1931).

The method of studying the fresh pancreas was as follows. Immediately after the removal of the pancreas from the body, pieces measuring about 0.5 mm. to 1 mm. in greatest dimension were cut off from several regions and

placed on a slide. A drop of normal saline was added and a coverslip applied. With pieces of tissue of this size, the weight of a No. 1 coverslip is sufficient to flatten the tissue without undue crushing or distortion. Upon examination with the microscope the specimen appears to be about the thickness of 3 to 5 acini. This thickness, admittedly, is not ideal and to some extent spoils the illumination; however, the conditions are adequate for good general examination, and the thin edge of the tissue is easily studied.

The dose of neutral red finally considered as optimum was approximately 0.0002 gm. per gramme of mouse body-weight. This is conveniently administered in a 1 per cent. solution, and it is sufficient for results that mice weighing 25 to 29 gm. receive 0.6 ml. and those weighing 30 to 34 gm. 0.7 ml., &c. The possibility must be kept in mind that a different batch of neutral red may require its own standardization. However, in view of the similar findings derived from the five batches tried in this study, it seems unlikely that the variation from 0.0002 gm. per gramme of mouse body-weight will be very great.

THE NEUTRAL RED GRANULE CYCLE

Fifteen minutes after the subcutaneous injection of the optimum dose of neutral red, the only change observed in the pancreas acinar cell is one of colouring. The cytoplasm, which is normally colourless, is now homogeneously light pink. Neutral red granules are not identified at this stage.

Half to three-quarters of an hour after injection, the cytoplasm is slightly darker pink and there are four to six small, separate, spherical, reddish granules about 0.7 μ in diameter scattered in the regions of the cytoplasm just basal to the zymogen granules and for a short distance along the sides of the nucleus. The neutral red granule is sharply demarcated from the surrounding, homogeneous, pale pink cytoplasm. It resembles very closely a drop of coloured oil in water. It is not birefringent. It is too small to permit one to draw detailed conclusions about its finer structure, and one can only say that it appears to consist of a fluid immiscible with the ground cytoplasm.

During the course of the next half-hour to three-quarters of an hour, there is a gradual increase in the number of the neutral red granules until there are 8-12 of them. During this period it is observed that some of the neutral red granules no longer occur as separate bodies but apparently have come together to form clumps or aggregates of two or three granules.

During the ensuing periods up to about 8 hours after injection, the process of aggregation becomes more marked (see fig. 1). Fewer single granules are seen and each aggregate appears to consist of about 4-8 granules; these are bound together by some unknown force, but do not fuse. Each aggregate resembles a small bunch of grapes. It is often not possible to count the number of granules in each aggregate with certainty, as some of them tend to obscure others. At late stages of aggregation each clump of granules tends to be somewhat elongated. The position of the neutral red granule aggregates is similar to that first observed for the single granules—in the region just

basal to the zymogen granules and along the sides of the nucleus. They are not observed mixed amongst the zymogen granules or immediately adjacent to the base of the cell.

Additional changes in the aggregates during the latter stages of development include an increase in intensity of staining and some further increase in size and number. At the time of their maximum development there may be 15–20 aggregates, each 2.5 to 3 μ in greatest dimension.

After about 8 hours there is a gradual decrease in number and size of the aggregates and discrete granules until at 12 to 15 hours after injection of neutral red, the cytoplasm is free of both elements and normal in appearance.

It should be added that if a larger dose of neutral red is injected, the only variation from the sequence of events just described is that the granules and aggregates are formed more quickly after injection and persist longer.

It is concluded, therefore, from the foregoing results that there is, as had been anticipated, a definite series of changes in the mouse pancreas acinar cell by which the neutral red granules appear and later disappear after the subcutaneous injection of a predetermined optimum dose of neutral red.

CRITIQUE OF THE METHOD USED IN STUDYING THE GRANULES

In any experiment in which a foreign substance is introduced into an animal, the possibility that the functioning of certain cells will be disturbed by that foreign substance must receive first consideration. Further, the possibility that what one observes in the cell after the introduction of a foreign material represents not the normal workings of the cell within the limits of its physiological potentialities but manifestations of disturbed functioning, must be kept in mind.

These were points of major consideration in the determination of the optimum dose of neutral red. In fact, the optimum dose finally decided upon was a direct result of such considerations.

Previous mention has been made of the fact that if very small doses of neutral red were injected the neutral red granules did not develop, but if very large doses such as 1 ml. of a saturated solution were given, pathological changes occurred. These changes, interpreted as pathological, were observed several hours after the administration of neutral red and took the form of large reddish vacuoles measuring up to 4 μ in diameter, filling the cytoplasm.

FIG. 1 (plate). Photomicrographs of living acinar cells of the pancreas of the mouse, 5 hours after the subcutaneous injection of the optimum dose of neutral red. The specimen consisted of a piece of tissue about 0.5 mm. across, placed in normal saline on a microscopical slide. (The neutral red granules of an acinus cannot all be in focus at the same time.)

A shows the number and size of the neutral red granule aggregates and their position in relation to the zymogen granules. The neutral red granules appear black in the photograph, the zymogen granules white.

B shows the position of the neutral red granule aggregates in the cell: they are basal to the zymogen granules. The apical borders of three cells are in focus in the centre of the acinus (at the site of the potential lumen).

C shows the irregular, finely nodular structure of the neutral red granule aggregates.

Were they not coloured, they would strongly suggest a state of pathological change in the cell commonly known as hydropic degeneration, that is, extensive vacuolization. The task at hand, then, was to find the dose of neutral red intermediate between these two extremes in which the neutral red granules could be observed under conditions in which the physiological stability of the cell was preserved. With this end in view, the following points were taken into consideration:

(1) The volume of the neutral red solution injected should not be excessively large in proportion to the size and weight of the animal and it would be an advantage if that volume were one which could be conveniently administered.

(2) The dose of neutral red should not be one which would make the animal obviously ill. The mice remained alert and active after the optimum dose had been injected. Such was not the case when large doses were administered, for the animals became lethargic and weak. It should be recorded, however, that when the optimum dose was given, the animals seemed disinterested in food until some 12 to 15 hours later, when the pancreas was cleared of neutral red.

(3) It was felt that some measure of safety could be afforded the cell if the dose was one in which it required several hours before maximum development of the neutral red granules was attained. It was considered that in this way the cell could perhaps gradually adjust itself to the presence of a foreign material, in contrast to a dose the size of which would cause sudden or maximum changes within the first hour or two after injection.

(4) The dose of neutral red should be one in which the complete cycle of neutral red granule formation and disappearance could be followed, but one by which neutral red did not remain in the tissues for an excessively long period. The possibility was entertained, but without evidence, that the presence of neutral red for an extended period might in some way injure the cell, and it was known that within 24 hours, if no food was taken, the general metabolism of the mouse would be upset as evidenced by the development of fatty changes in the liver. Accordingly, it was felt that 12 to 15 hours represented the safest length of time during which the neutral red granules could be studied.

(5) The dose should be one which did not occasion either immediate or late harmful effects. The immediate effects of the optimum dose have been discussed above (2). In an attempt to discover the presence of any late effects, animals were observed for a month after the injection of neutral red. They were completely normal in all respects.

The foregoing may be regarded as reasonable measures for preserving the normal functioning of the cell, but it was felt that evidence of a more objective nature should be secured indicating to what degree, if any, the general physical and chemical balance of the cell had been disturbed by the presence of neutral red.

It is generally agreed that mitochondria are very susceptible to alterations in cytoplasmic conditions. Thus, after exposure of pancreatic tissue to certain

fixatives, or when mitochondrial fractions from homogenized pancreas are suspended in hypotonic solutions, the mitochondria fail to retain their natural rod-like structure but assume spherical, globular, or gourd-like shapes. Such forms are considered by cytologists to be abnormal.

It was therefore concluded that a study of the mitochondria during the several stages of neutral red granule formation after the injection of neutral red, would provide information as to whether the precautionary measures previously discussed were of value and whether the optimum dose of neutral red caused any serious alteration in the normal functioning of the pancreas acinar cell.

Two methods of approach to this problem were employed, namely, the classical Altmann technique for showing mitochondria, and the immersion of bits of fresh pancreas for 15 minutes in a 1:500 solution of Janus green. Both methods showed apparently normal rod-like mitochondria in the pancreas after injection of neutral red; the mitochondria resembled those in the uninjected control.

It is concluded from these findings that the administration of neutral red in the dosage finally determined as optimum does not upset the normal dynamics of the pancreas acinar cell to a degree which can, at the present time, be shown cytologically. Therefore, it is submitted that until the time when more sensitive methods for examination of cellular stability are available, the fairest interpretation of the present results is that the cycle following the injection of the optimum dose of neutral red in which neutral red granules form in and disappear from the cell, represents, not necessarily the normal functioning of the cell, but a process within the physiological capabilities of the cell.

THE ORIGIN AND NATURE OF NEUTRAL RED GRANULES

It is necessary first to ask whether the neutral red granules and aggregates represent pre-existing vacuoles in the cell which are coloured when in contact with the dye, or whether they are new formations, objects not normally present in that form and position in the cell, but produced by the entry of neutral red into the cell.

It is important that this question should be asked about each type of cell whenever neutral red is used, for it is known that in some cells, such as ganglion or liver cells, there are vacuoles readily visible in the fresh, unstained state as clear, colourless vacuoles, which become red after the immersion of the tissue in a solution of neutral red; because of their shape and size they qualify for the name 'neutral red granules'. In other tissues, in which red granules develop after injection of neutral red, similar vacuoles are not apparent in the unstained cell, and one is left to conclude that in the second type of tissue, the neutral red granules clearly visible after the injection of neutral red are new formations produced by the presence of neutral red in the cell. To these two must be added the third possibility that the neutral red granules seen after injection of neutral red represent a combination of both pre-existing and newly formed vacuoles.

It is the author's opinion that the mouse pancreas acinar cell falls in the second group, i.e. that the neutral red granules are new formations. This conclusion is based on the following studies and considerations:

(1) The positions of the neutral red granules and aggregates have from the study of the neutral red granule cycle been established as being confined to the regions just basal to the zymogen granules and for a short distance along the sides of the nucleus. Further, these are the only sites in which the neutral red granules are seen; they are not observed mixed amongst the zymogen granules, or basally in the cell immediately adjacent to the basement membrane. Routine microscopic examination of these regions of the cell in tiny bits of normal unstained pancreas discloses a homogeneous cytoplasm. This is 'empty' except for the ends of mitochondria, and sharply demarcated from the prominent zymogen granules.

(2) Pre-existing vacuoles are readily visible by ordinary microscopy in the liver cell, and upon staining with neutral red certain ones are not unlike the neutral red granules seen in the pancreas. The zymogen granules in the pancreas have the appearance of vacuoles, and they are the most prominent component in the unstained living cell. The author finds it impossible, therefore, to accept the statement that there are vacuoles normally present in the unstained pancreas acinar cell in the number and of the size of the neutral red granules and aggregates. As previously described, the neutral red granules and aggregates in the advanced stage of development occupy a major portion of the cytoplasm just beneath the zymogen granules and the aggregates measure up to 2.3μ in greatest dimension. Vacuolar structures of this size should be easily visible.

(3) The application of phase-contrast microscopy was found to be impossible when 1 mm. pieces of tissue were used, because the thickness of the specimen caused excessive diffusion of the light. In subsequent attempts to secure specimens of pancreas suitable for phase study, tissue was homogenized in an all-glass tube, and although small clumps of acinar cells were obtained, these showed too much distortion of cellular architecture to be considered of value. Finally, the method of teasing bits of pancreatic tissue in normal saline with sharp needles provided suitable preparations for study. Examination of these cells failed to show vacuoles or aggregations of vacuoles above or beside the nucleus, that is to say in the positions occupied by the neutral red granules or aggregates after the administration of neutral red.

(4) There is still another method by which the presence of vacuoles stainable with neutral red can be ascertained. This is the commonly used procedure of simply immersing bits of tissue in a solution of neutral red. Presumably, as in the liver and ganglion cells in which pre-existing vacuoles may be identified and which are coloured by neutral red, similar vacuoles if present in the pancreas should become stained and apparent after immersion of this tissue in neutral red. Accordingly, two experiments were carried out. Small pieces of pancreas measuring 0.5 mm. to 1 mm. were divided, some being placed in a 0.001 per cent. solution of neutral red in normal saline,

others in a 0.001 per cent. solution of neutral red in distilled water for periods up to 3 hours.

The results of this study showed that in the specimens of pancreas immersed in the solution of neutral red in distilled water, although there was extensive diffusion with resulting pinking of the tissue, this colouring was homogeneous, affecting all the cell components: at no time were neutral red granules observed. In contrast, the bits of pancreas which were treated similarly in a solution of neutral red in normal saline showed many neutral red granules. Although the organization of these was never as neat as in tissue from injected animals, their character and distribution were interesting.

In the inner portions of the specimen, the neutral red granules were small and separate, occupying roughly the area of the cytoplasm just basal to the zymogen granules and to the sides of the nucleus. In areas lying roughly intermediately between the inner portion and the edge of the specimen, separate granules and clumps of granules similar to aggregates were observed. At the very edge of the specimen the elements stained with neutral red were in the form of large vacuoles scattered throughout the cell. These last were in no way dis-similar to the structures previously described as pathological and observed in the pancreas of an animal after injection with a large dose of neutral red.

These findings are interpreted in the following manner:

(1) It is assumed that the cells placed in a distilled water solution of neutral red did not live long. If pre-existing vacuoles stainable with neutral red were present they should have been apparent, since there was ample diffusion of neutral red into the tissue.

(2) On the other hand, it is assumed that the cells placed in neutral red in normal saline lived for a considerable period and because of this, conditions were to some extent comparable to those in the pancreas of the living animal. It is therefore concluded that the living cell detached from the animal in contact with neutral red has the same capacity for neutral red granule formation as the acinar cell in the animal injected with neutral red.

In addition to those workers who feel that the neutral red granules represent pre-existing vacuoles present in the normal cell, there are those who hold that still another component of the cell forms the neutral red granules. Bensley (1911) and Gatenby (1931) have stated that the neutral red granules are simply the prozymogen or immature zymogen granules which have been stained by neutral red. In view of this possibility it was felt necessary to repeat their studies by methods similar in principle to those they adopted. Mice were placed in metal cages without food (but with water) for a period of 24 hours; both starved and control (feeding) animals were then given a subcutaneous injection of the optimum dose of neutral red. Six hours later, food still being withheld from the experimental animals, both groups of animals were killed and the pancreas examined microscopically.

It was the contention of Bensley and of Gatenby that in the 24-hour period in which no food was ingested, there would be a maturation of all the pro-

zymogen granules in the pancreas, leaving none to be stained by neutral red. In their experiments (Bensley using guinea pigs and Gatenby the newt *Triturus* (= *Diemyctalus*), they reported that in the starved pancreas neutral red granules were not present whereas they were observed in the pancreas of a feeding animal. They concluded from this that the neutral red granules were actually the prozymogen granules.

We were unable to confirm these findings of Bensley and Gatenby in our experiments with the mouse, the pancreas of both groups showing large numbers of neutral red granule aggregates as they had been seen many times before during the study of the neutral red granule cycle.

Although the possibility of a species-difference must be recognized, we have concluded from our experiments that in the mouse it is not the prozymogen granules that form the neutral red granules.

The contention of Hirsch (1939) that at least the Golgi 'pre-substance' is stainable with neutral red, is one which the present author does not feel qualified to discuss. Although 8 months of the writer's time was devoted to a study of the secretion cycle in the pancreas, his results in this line were unsatisfactory, and definite conclusions concerning the Golgi system during the secretion cycle as described by Hirsch were never reached. Although a definite statement must be deferred for the present, the author is strongly inclined to believe from data which will be presented later, that the neutral red granules observed in the present study are not pre-substance stained by neutral red.

There is one final possible source for vacuoles, which, to the author's knowledge, has not been previously advanced. It is conceivable that there exist in the pancreas cell small granules which cannot be differentiated from the zymogen granules, and that these, when in contact with neutral red, go on to form the neutral red granules. In such a hypothesis, however, one would expect to observe neutral red granules mixed amongst the zymogen granules, but they are not. Further, this hypothesis would imply a movement of such granules from the region of the zymogen granules to those parts of the cell where the neutral red granules and aggregates are found, as previously described. At no time was evidence encountered to support such a possibility.

It is concluded, then, from the foregoing considerations and experiments that the neutral red granules as observed after the injection of neutral red, are not stained pre-existing vacuoles normally present in the cell, but that they are new formations produced by the entrance of neutral red into the cell.

It has been said by some investigators (Covell, 1928; Beams, 1930), that neutral red granules are seen scattered amongst the zymogen granules, and it has been suggested that the neutral red granules are extruded into the lumen of the acinus along with the zymogen granules, and in that way the neutral red is removed from the cell.

The author does not consider that neutral red granules are present in the region of the zymogen granules. On numerous occasions during the study of

the pancreases of animals injected with neutral red, the initial impression was that the neutral red granules were mixed amongst the zymogen granules; but when the object was carefully focused so that the majority of the zymogen granules of a single cell were sharply in view, no neutral red granules could be identified. This observation suggests that when the zymogen granules of one acinus are not in sharp focus, the neutral red granules which appear to be among the zymogen granules actually belong to an over- or underlying cell of another acinus. This may have been the mistake made by previous

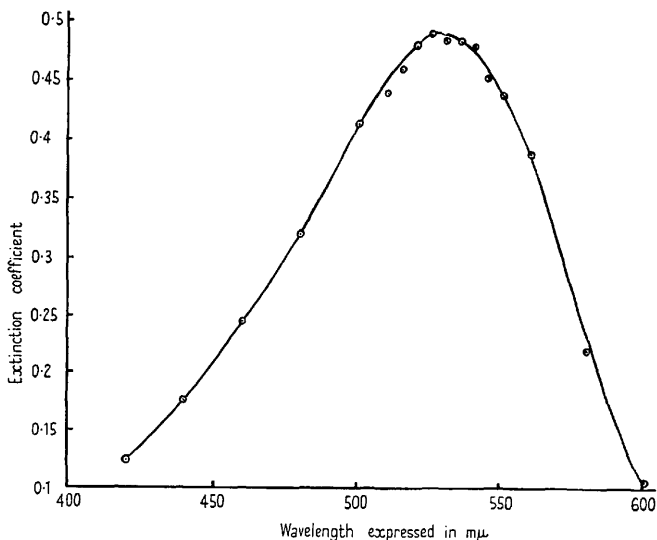


FIG. 2. Graph showing the absorption of light of different wave-lengths by an acid solution of neutral red.

investigators who claimed that neutral red granules were observed in the region of the zymogen granules.

THE EXCRETION OF NEUTRAL RED

It was observed during previous experiments that after the injection of neutral red, the urine was red not only during the 12- to 15-hour neutral red granule cycle observed in the pancreas, but also until about 48 hours after injection. It was concluded, therefore, that urinary excretion was at least one method by which neutral red was removed from the body, and it was felt that some information concerning the fate of the neutral red in the body might be revealed by a comparative study of the amount of neutral red

injected with that excreted in the urine. It was felt that if all or nearly all the neutral red injected were recovered from the urine, this result would prove that the neutral red is unchanged when in the neutral red granules. If, on the contrary, much less than the total amount were recovered, this would suggest that the neutral red might be broken down while in the granules. It was recognized, of course, that the breakdown might occur in some organ other than the pancreas (e.g. the liver).

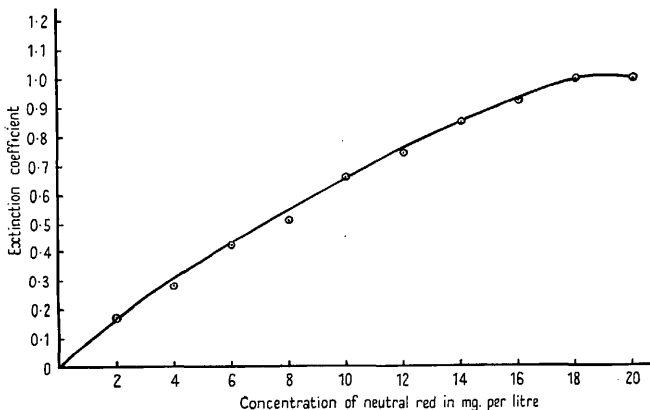


FIG. 3. Graph showing the absorption of light of wave-length 525 $m\mu$ by an acid solution of neutral red at different concentrations

Below pH 6 the dye is red. The extinction coefficient at various wave-lengths, at this pH, is shown in fig. 2. It will be noticed that the maximum absorption is at a wave-length of about 525 $m\mu$. In fig. 3 the extinction coefficient with light of this wave-length is plotted against various concentrations of neutral red in aqueous solution at pH 6. This graph may be used to determine the concentration of neutral red in any solution of the dye. It is only necessary to measure the extinction coefficient of the specimen of unknown concentration, and the concentration of the dye can then be read off from the graph.

This method was used to measure the amount of neutral red in the urine of injected mice. Groups of two or three adult mice of similar weights were injected with the optimum dose of neutral red and placed in metabolism cages, from which all urine was collected. The urine specimens were made up to a volume of 250 or 500 c.c. according to the amount of washing of the cage necessary to recover all the urine. The specimens were acidified and colorimetric readings were taken. This was done at 24 hours and 48 hours after injection. Longer periods than this were not necessary, because earlier

experiments had shown that after 48 hours too little neutral red was excreted to give colorimetric readings.

Table 1 shows the results of these studies.

TABLE 1

<i>Expt. No.</i>	<i>No. of animals</i>	<i>Amount injected (mg.)</i>	<i>Amount recovered in 1st 24-hr. period (mg.)</i>	<i>Amount recovered in 2nd 24-hr. period (mg.)</i>	<i>Total amount recovered in 48 hrs. (mg.)</i>	<i>Percentage recovered in urine</i>
1	3	21	5.625	0.36	5.99	28.0%
2	2	16	4.30	0.23	4.53	28.0%
3	3	24	5.45	1.20	6.65	27.7%
4	3	21	5.10	0.55	5.65	26.9%
5	3	21	5.45	0.34	5.79	27.6%
6	3	21	5.75	0.69	6.44	30.7%

These results indicate that only a little more than one-quarter of the neutral red injected is excreted in the urine as neutral red. After 48 hours no part of the body appears to be coloured red, and it is therefore unlikely that storage of unaltered neutral red occurs. Since examination of the faeces of these animals failed to show the gastro-intestinal tract as the route by which neutral red is removed from the body, the two main avenues by which foreign or toxic substances are discarded from the body have been excluded. It is therefore concluded that the major portion of a given dose of neutral red is altered within the animal body. The exact site and the method by which this is achieved have not been determined.

COMMENT

It will be observed that no attempt is made in the present study to relate findings to function in the pancreas, although several interesting possibilities of what is going on in the neutral red granule are considered. In what ways, if any, these may reflect the normal everyday secretion processes in the pancreas acinar cell are additional problems to be explored. At this point it should be said that in older research with neutral red the practice of assuming that findings with one type of cell were true for others was a fundamental error and contributed heavily to the confusion associated with the Golgi problem. The author wishes to emphasize that the studies reported here were confined to the pancreas acinar cell of the mouse. Whether similar neutral red granule cycles may be shown in other tissues or other species must await further research.

ACKNOWLEDGEMENTS

I wish to thank Dr. John R. Baker, without whose help and stimulating discussions these studies would not have been done. Additional thanks are due to Prof. A. C. Hardy, F.R.S., who kindly provided facilities for this research, Dr. Frank Billett, Ph.D., A.R.I.C., for assisting me with the colorimetric determinations, and Miss B. M. Jordan for helping in photomicrography.

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