CHEMICAL STUDIES ON TUMOR TISSUE

IV. The Staining with Neutral Red of Fresh Preparations of Mouse Tumor Cells

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Introduction

In studying the chemical properties of individual tumor cells *in vitro,* it was desirable to know whether the cells under observation were alive or dead; it was also desirable to determine whether the living cells in any given experiment died during the course of observation. Of the various criteria of cell viability—*e.g.,* motility, phagocytosis, fertilization, cell division, vital staining, etc.—which have been employed in the study of various types of single cells, the one selected as most suitable for the purposes of this study was vital staining.

The staining with vital dyes of cells of mammalian tissues both *in vivo* and *in vitro* has been investigated by many workers. There is general agreement that vital dyes stain the nucleus in dead cells or in severely injured cells. As regards the living cell, however, the findings have not been so uniform (1–5).

In the case of neutral red, it has come to be widely accepted that cells treated with this dye often respond as follows. (*a*) In the living cell the dye is seen in localized areas (granules, vacuoles) in the cytoplasm while the rest of the cytoplasm, as well as the nucleus, appears colorless (6–10). (*b*) As a cell dies the granules and vacuoles lose their stain, and the nucleus and the cytoplasm become diffusely red (7, 8, 11, 12). (*c*) In the dead cell the entire nucleus is stained diffusely red and, in addition, the cytoplasm also is usually stained diffusely red (7, 11, 14–16).

Although many types of cells react in this fashion with neutral red, other types do not. As various observers (40, 2, 5, 16–28) have noted, cells of one tissue may exhibit numerous stained cytoplasmic bodies, while cells of another tissue may exhibit few stained bodies or none at all. Furthermore, cells of the same tissue under identical conditions may exhibit this variation in response to the dye. These differences in staining behavior are discussed below.

In the investigation reported here fresh mouse tumor tissue was immersed in salt solutions containing neutral red. Observations were then made on the nature of the staining in order to see whether the cellular reaction to neutral red described above was applicable as a criterion of viability in the case of these cells under these experimental conditions.

From the start it was obvious that the two types of cells predominating in these mouse sarcomas gave a strikingly different response to neutral red. The overwhelming majority of one type of cell stained promptly and showed

the typical vital staining reaction, whereas the overwhelming majority of the other type of cell failed to show any stain whatever. Quantitative data were thereupon secured for both types of cells, on the relative numbers which showed localized [1] staining of the cytoplasm, stained nuclei, or no stain at all.

Most of the observations in this investigation were made on mouse sarcoma 37 which, under these experimental conditions, appears to consist chiefly of two types of cells: (*a*) a spindle cell with a large heavy-walled nucleus, containing a variable number of prominent nucleoli; (*b*) a " round " cell, with nucleus barely visible in the living state (29).[2] Initially, most of the " round " cells were seen to exhibit the localized type of staining; a few were unstained, and a few had stained nuclei. In the course of a few hours the " round " cells with stained nuclei increased in number with a concomitant decrease in cells showing localized cytoplasmic staining.

On the other hand, the majority of the spindle cells under identical conditions regularly failed to show any stain. A few of these cells exhibited localized staining in the cytoplasm, and a few showed diffusely stained nuclei; but most of them appeared completely colorless. The number of colorless cells decreased rapidly in the course of a few hours with a concomitant increase in the number of cells with diffusely stained nuclei.

Several series of such observations were made at each of 3 different temperatures: at 38° C., at room temperature (approximately 23° C.), and at cold room temperature (approximately 8° C.). Similar observations were also made with cells of a few normal tissues of the mouse.

Technic

Healthy tumor tissue was minced with fine scissors in a few cubic centimeters of salt solution containing neutral red. After a few minutes some of the minced tissue was transferred with a fine pipette to a slide, covered with a slip, and sealed. These preparations, or spreads, were studied with an oil immersion lens at a magnification of 1100 diameters. Sometimes a given sealed slide was under observation for many hours. At other times, depending upon the purpose of the experiment, fresh slides were prepared at intervals from the minced tissue remaining in the original dye solution.

Although most of the observations reported here were made upon cells of mouse sarcoma 37, some observations were also made using transplants of sarcomas originally induced in a mouse with 1:2:5:6-dibenzanthracene (30). Similar observations were also made upon normal mouse testis and spleen.

The solutions employed all had a salt concentration of 155 milli-equivalents per liter. In some cases, the solutions contained only NaCl, except for 1 mM of Na_2HPO_4 as buffer. In other cases, the balanced salt solution of Shear and Fogg (31) was employed. In most cases, however, the solution used was the balanced salt solution containing, in addition, 100 mg. glucose per 100 c.c.

[1] The term " localized " staining is used in this paper to include granular staining, vacuolar staining, and staining of indeterminate small bodies in the cytoplasm.

[2] No attempt was made to identify cytologically the cells classified in these two groups. For a discussion of this subject see " Sarcoma Cells " by W. H. Lewis (10). The cytology of mouse sarcoma 37 has been studied by Ludford (2, 53). According to Lewis (personal communications) the spindle cells are malignant, and the " round cells correspond undoubtedly to what we are quite sure are monocytes and macrophages."

EXPERIMENTAL

Preliminary Observations: The great majority of the cells of mouse sarcoma 37, as well as of several other mouse sarcomas similarly examined, were grouped into the two classifications described above, *i.e.*, round and spindle. Cells which could not be classified in this way were few in number and were not considered in this study.

Upon immersion of minced tumor tissue in salt solutions containing neutral red, the round cells stained promptly and vividly, exhibiting the type of localized cytoplasmic staining considered characteristic of living cells. On the other hand, the spindle cells for the most part failed to show any staining at all. This striking difference in reaction towards neutral red was found to be independent of the nature of the salt solution and of the concentration of dye.

Since most of the spindle cells failed to exhibit staining when dilute solutions of neutral red were employed, higher concentrations were used in an effort to promote staining of these cells. It was only when the dye concentration was increased to the point where it produced toxic effects (1:20,000) that any considerable number of the spindle cells became stained. Under these conditions, however, diffuse staining of nucleus and of cytoplasm was observed for the most part; only rarely was localized staining of the cytoplasm obtained. A dye strength of 1:40,000 was selected as yielding the greatest number of cells with localized cytoplasmic staining. Even at this concentration, however, the great majority of the spindle cells remained unstained upon immersion in dye solution, although the round cells stained at once. Furthermore, the few spindle cells which exhibited localized staining contained for the most part a smaller number of stained areas per cell than was the case with the round cells.

The occasional spindle cell which exhibited localized staining was often seen to lose the stain, the cell becoming entirely colorless in one to two hours. After several hours in the decolorized condition, such cells became diffusely stained. In this sequence of events, the colorless state was intermediate between the localized cytoplasmic staining and the diffuse staining of nucleus and cytoplasm.

Quantitative Observations: The foregoing impressions were borne out when comparisons between the two types of cells were made on the basis of quantitative data. Many thousands of round and spindle tumor cells were examined individually, and the type of staining noted in each case. The procedure was as follows:

Spreads were made as described previously. Several hundred cells of one type (either round or spindle) were then observed individually and placed in one of the following three categories: (*a*) localized cytoplasmic staining; (*b*) diffuse nuclear staining; (*c*) absence of staining. For this classification, both isolated cells and marginal cells of tissue fragments were included. The percentage of the total number of cells falling in each of these three categories was then computed.

Using the same spread, similar data were then obtained for the other cell type. These quantitative observations confirmed the original impressions as

TABLE I: *Kind of Staining Obtained After Immersion in Neutral Red Solution for One-half Hour at 38° C.*

Cell Type	Localized Cytoplasmic Staining	Colorless	Diffuse Nuclear Staining
Round	80%	12%	8%
Spindle	7%	85%	8%

regards the difference in staining reaction exhibited by round and spindle cells toward neutral red. At 38° C., the distribution of the cells in the three

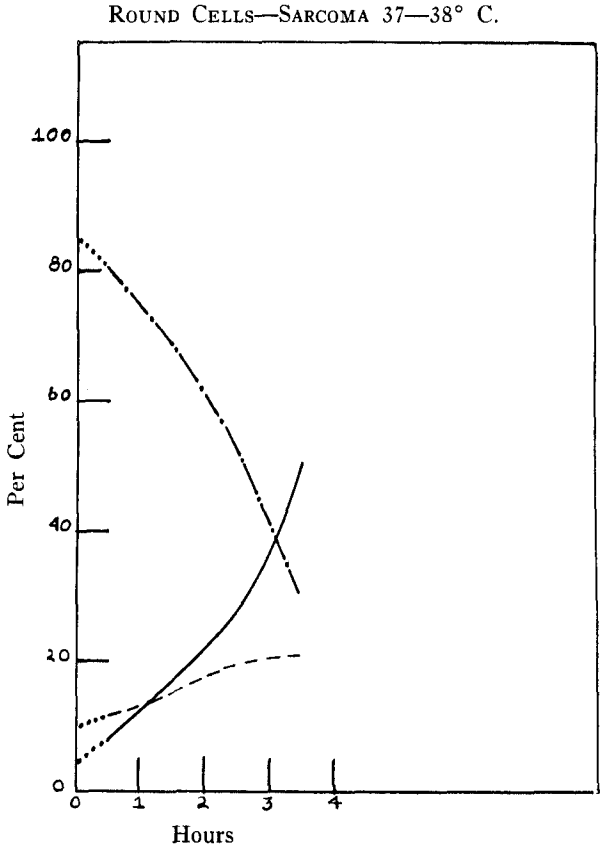

FIG. 1. DISTRIBUTION OF NEUTRAL RED STAINING TYPES AMONG ROUND CELLS OF MOUSE SARCOMA 37 AT 38° C.

—·—·— Localized cytoplasmic stain. ———— Diffuse nuclear stain. ------ Colorless. ····· Extrapolated.

staining categories, one-half hour after mincing in the neutral red solution, was as shown in Table I.

As can be seen from this table, the two types of cells showed a distinct difference in behavior towards neutral red.

Effect of Immersion Time at 38° C.: Since it was considered possible that prolongation of immersion time might affect the nature or intensity of the staining, counts were made at intervals, employing each time a spread freshly

prepared from the original minced tissue remaining in the dye solution. The results obtained at 38° C. are shown in Figs. 1 and 2. The abscissae in all the figures give the time in hours subsequent to the mincing of the tumor tissue in dye solution. The ordinates give the relative numbers of cells showing the three types of reaction to neutral red.

Fig. 1 gives the composite curves obtained for the round cells on averaging the results of seven experiments in which such counts were made at 38° C. Although there was some variation from experiment to experiment in the positions of the curves relative to the ordinate axis, their shapes and slopes

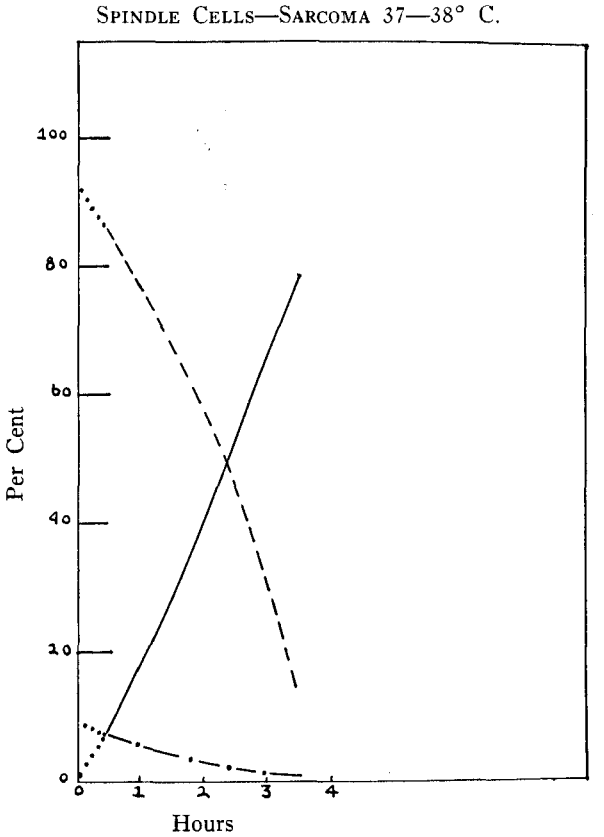

FIG. 2. DISTRIBUTION OF NEUTRAL RED STAINING TYPES AMONG SPINDLE CELLS OF MOUSE SARCOMA 37 AT 38° C.

—·—·— Localized cytoplasmic stain. ———— Diffuse nuclear stain. ------ Colorless. ····· Extrapolated.

were essentially the same. The variations of the individual curves from the composite curve were within 20 per cent. Fig. 2 gives the analogous results obtained for the spindle cells in these same seven experiments. Approximately 4000 individual round cells and an equal number of individual spindle cells were classified.

The trends of these composite curves may be characterized as follows:

The Round Cell (Fig. 1): The dot-dash line represents the percentage of all cells which exhibited localized staining in the cytoplasm. At the first

ROUND CELLS—SARCOMA 37—23° C.

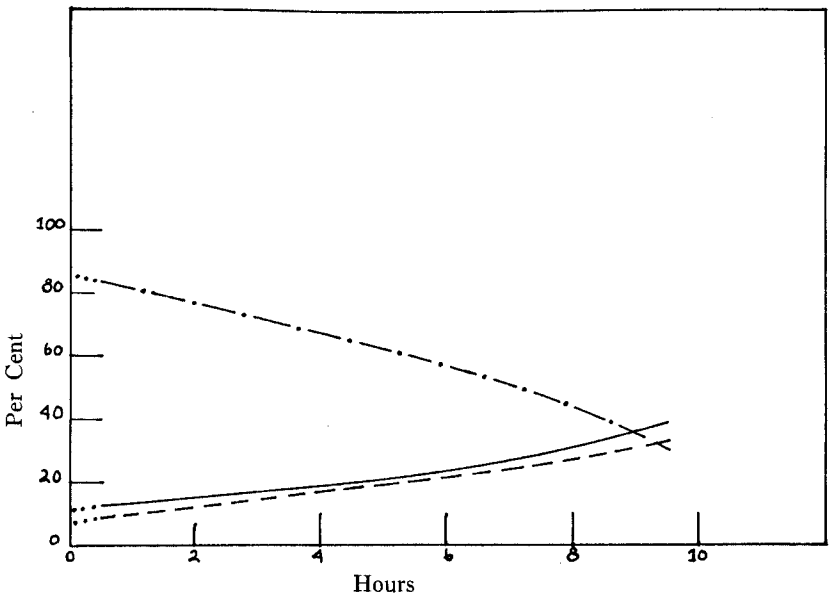

FIG. 3. DISTRIBUTION OF NEUTRAL RED STAINING TYPES AMONG THE ROUND CELLS OF MOUSE SARCOMA 37 AT 23° C.

—·—·— Localized cytoplasmic stain. ———— Diffuse nuclear stain. - - - - - - Colorless. ····· Extrapolated.

SPINDLE CELLS—SARCOMA 37—23° C.

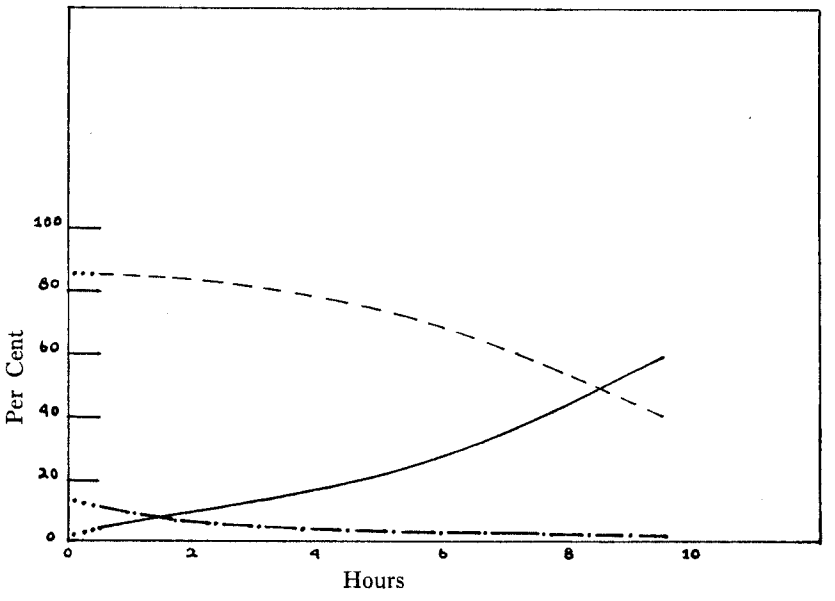

FIG. 4. DISTRIBUTION OF NEUTRAL RED STAINING TYPES AMONG SPINDLE CELLS OF MOUSE SARCOMA 37 AT 23° C.

—·—·— Localized cytoplasmic stain. ———— Diffuse nuclear stain. - - - - - - Colorless. ····· Extrapolated.

reading, one-half hour after mincing, most of them (80 per cent) were stained in this way. The percentage dropped off rapidly until, after three and a half hours, only 30 per cent of the cells showed localized staining.

The continuous line represents the proportion of cells which had stained nuclei. At the first observation, only about 8 per cent had stained nuclei. This percentage increased rapidly until it reached a value of 50 per cent at the end of three and a half hours.

The broken line represents the proportion of cells which had no discernible stain. At the first reading these cells constituted about 12 per cent of the total. This percentage increased slowly to a maximum of about 20 per cent.

The Spindle Cell (Fig. 2): In contrast to the round cells, the percentage of spindle cells that had localized staining was small, being only about 7 per cent at the first reading. The percentage decreased gradually to practically zero in the course of three and a half hours. The large majority (85 per cent) of the cells at the beginning showed no visible stain. Their number fell off rapidly to a value of about 15 per cent in the course of three and a half hours. Concomitantly, the number of cells with nuclear staining increased from 8 to about 80 per cent.

In these two sets of curves it is seen that the colorless spindle cells had the same distribution and the same slope as the round cells with localized cytoplasmic staining (*i.e.*, the so-called "vital staining").

Effect of Temperature: The experiments described above were performed at 38° C. Similar experiments were performed at room temperature (approximately 23° C.) and at cold room temperature (approximately 8° C.).

The data for the experiments at room temperature are summarized in Figs. 3 and 4. Each set of curves, like those in Figs. 1 and 2, represents the average of 7 experiments. The trends shown by the curves at room temperature are quite similar to those shown at 38° C. The notable difference is the more gradual slope. This is due to the fact that the changes in staining behavior occurred more slowly at the lower temperature.

Figs. 5 and 6 give the results of analogous experiments carried out at cold room temperature (approximately 8° C.). Each set of curves represents the average of two experiments. In general, the trends are the same as at higher temperatures. Again, the chief difference is in the slopes of the curves. At this low temperature the curves sloped so gradually that the scale was changed; each unit on the abscissae is ten times as great as in Figs. 1 to 4.

It should be noted that at all temperatures the majority of the round cells initially had localized staining in the cytoplasm, while the majority of the spindle cells initially were colorless. At all temperatures, for both types of cells, the nuclear stained cells constituted only a small fraction of the total. As the proportion of round cells with localized staining, or of spindle cells with no staining, decreased, the proportion of cells with stained nuclei increased. The chief effect of lowering of the temperature was a slowing down of the rate of these changes.

Similar experiments were done, in duplicate, employing a sarcoma originally induced by 1:2:5:6-dibenzanthracene in a Strain D mouse. The results

ROUND CELLS—SARCOMA 37—8° C.

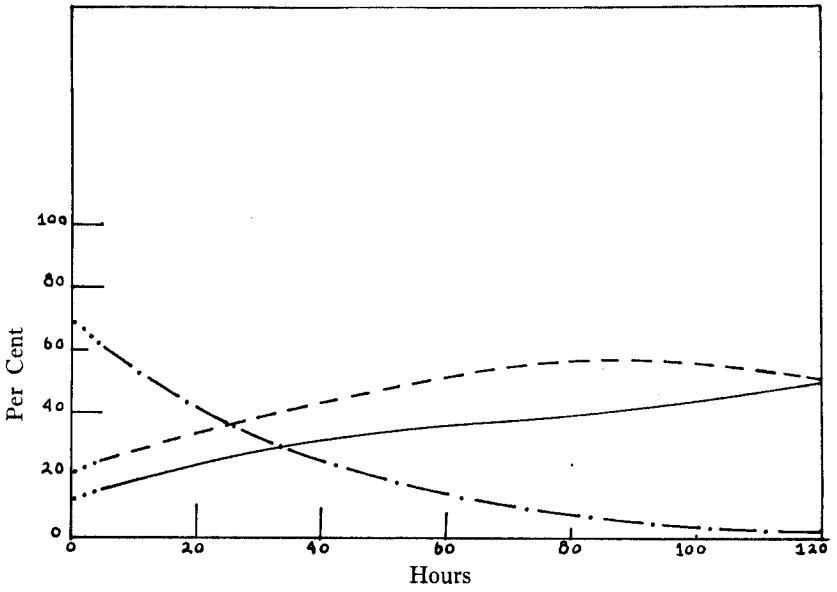

FIG. 5. DISTRIBUTION OF NEUTRAL RED STAINING TYPES AMONG THE ROUND CELLS OF MOUSE SARCOMA 37 AT 8° C.

—·—·— Localized cytoplasmic stain. ———— Diffuse nuclear stain. ------ Colorless. ····· Extrapolated.

SPINDLE CELLS—SARCOMA 37—8° C.

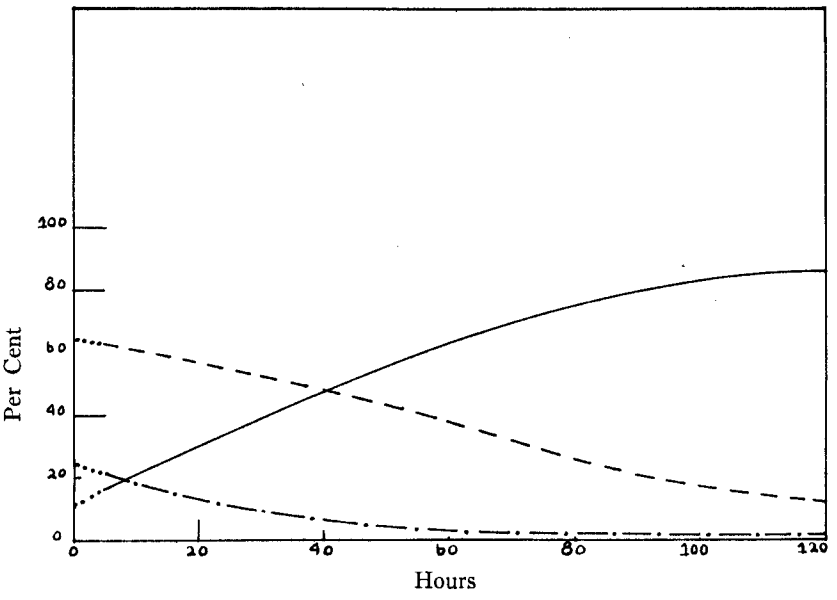

FIG. 6. DISTRIBUTION OF NEUTRAL RED STAINING TYPES AMONG THE SPINDLE CELLS OF MOUSE SARCOMA 37 AT 8° C.

—·—·— Localized cytoplasmic stain. ———— Diffuse nuclear stain. ------ Colorless. ····· Extrapolated.

obtained at 8° C. with this tumor paralleled those given for sarcoma 37 in Figs. 5 and 6.

Normal Tissues: Similar experiments were done with testicular and with splenic tissue of the mouse. No attempt was made to differentiate between the various types of cells present in these organs, *i.e.*, the cells were classified on the basis of their staining behavior without regard to their morphological characteristics.

Curves were made for each of these two normal tissues from the data obtained in cell counts, in the same manner as described above. At all temperatures, the curves for the cells of spleen and of testis were quite similar to those obtained for the round cells of sarcoma 37.

Fading: From time to time individual spindle cells which contained a large number of well stained granules were seen to lose the stain gradually, and finally to become completely colorless. During this process the intensity of staining of the red bodies became progressively fainter and their number fewer. This fading continued until, after several hours, all color completely disappeared.

Fading appears to take place in the round cells also. It can be seen from the slopes of those curves in Figs. 1, 3, and 5, which represent the proportion of colorless cells, that, as the number of cells with localized staining decreased, there was a concomitant increase in the number not only of nuclear-stained cells but also of colorless cells (*i.e.*, the broken-line curves).

This fading does not appear to be ascribable to pH change, for the cells which are colorless are distinguishable from cells which are yellow because of alkaline reaction. The disappearance of the color, therefore, is apparently due not to a shift in pH to the alkaline side but to some other mechanism. What this mechanism is can only be surmised at present. It may possibly be related to oxidation-reduction processes going on within the cell.

Certain color changes were noted which may bear on this point. When HCl was added to the solution containing such colorless cells, they promptly exhibited intense red staining throughout the nucleus and cytoplasm. When, however, minced tissue (either tumor or normal) was allowed to remain in the dye solution in a small Petri dish for twelve hours or more, the development of a fluorescent yellow color in the solution was occasionally noted. Addition of HCl to this fluorescent yellow solution did not cause a shift in color to the red. These two changes, *i.e.*, to the colorless form and to the fluorescent form, which neutral red underwent when in contact with tissue, resemble the changes to the leuco form and to the fluorescent form which Clark and Perkins (32) noted in a chemical study of this dye.

If neutral red is reduced to a leuco form within the cells under these conditions, this reduction is apparently not due to lack of oxygen. Although the readings were made with sealed slides, the spreads were always freshly prepared immediately before each reading from the tissue in the stock solution which was in equilibrium with air in the Petri dish. Each reading took only about thirty minutes.

That this brief period was not sufficient to exhaust the oxygen supply in the slide was shown by analogous experiments with methylene blue. When the tissue was first sealed on the slide, some of the cells were blue and others

were colorless. Upon continued observation of the same sealed slide, an increase in the number of cells stained blue was noted from hour to hour. Subsequently, as the oxygen supply progressively diminished, the blue color began to fade out of the cells until finally they were all colorless except for some cells situated near the seal. It was not until several hours had elapsed, however, that this decolorization began to be noted. That the fading was due to oxygen lack was shown by the prompt reappearance of the blue color upon breaking the seal.

Since several hours were required for the lack of oxygen in the sealed slides to become sufficiently pronounced to result in reduction of methylene blue, it would appear that the diminution in the oxygen content which occurred during the half hour required for the cell counts was not great enough to be responsible for reduction of neutral red to the leuco form within the cells. In addition, saturation with oxygen of the neutral red solution containing the minced tissue, immediately before the preparation of each slide, had no appreciable effect in preventing decolorization.

Furthermore, in other experiments with neutral red in which the same sealed slide was under observation for many hours, the cells which were colorless, either from the start or as the result of fading of localized staining, were invariably seen subsequently to become stained diffusely red. This took place in many cells after periods as long as twelve hours following sealing. Obviously in the case of neutral red the diminution of the oxygen supply was not sufficient to prevent development of red stain.

Finally, it may be pointed out that incidental observations of fading of neutral red have been made by other investigators using a variety of organisms and cells under conditions of plentiful oxygen supply (33, 17, 34, 11, 8, 12).

Lack of oxygen, therefore, does not seem to be the factor responsible either for the fading of neutral red from the stained localized areas in the cytoplasm of these mammalian cells or for the apparent absence of dye in the colorless cells.

Discussion

Although cells which contain neutral red stained bodies in the cytoplasm are usually referred to as " vitally stained," it was considered preferable, in describing the experimental observations of the present study, to employ the circumlocution " localized cytoplasmic staining " in order to avoid implications of viability.

Since the earliest days of the use of the so-called vital dyes, cells exhibiting this type of staining have been referred to as " vitally stained " and have been regarded as living cells (see page 1). As the result of more recent observations, however, the question may be raised as to whether the presence of so-called vital staining with neutral red necessarily demonstrates that the cell is alive. Furthermore, the question may be raised as to whether nuclear staining necessarily indicates cell death. Finally, cells which do not exhibit any stain at all do not fit into either category; opinions differ as to whether such colorless cells are dead or alive.

For example, Koehring (28) has reported that cells vitally stained with

neutral red could be killed by heat without causing the disappearance of color from the stained bodies in the cytoplasm, provided the temperature was kept below a certain critical point. If such dead cells were classified, without knowledge of their previous history, solely on the basis of the presence of neutral red stained bodies in the cytoplasm, the classification of these cells as alive would be erroneous.

An analogous situation appears to exist as regards nuclear staining. A rather large number of investigators have found that staining of the nucleus with neutral red, which usually is held to indicate death or irreversible injury to the cell, can occur in living cells under certain conditions. von Moellendorff (54), Alexandrov (35) and Nassonov (36) have summarized the results of many investigations on various types of animal cells in which it was found that nuclear staining was consistent with life as manifested by motility, cell division, etc. That plant cells, also, are capable of undergoing cell division while the chromatin material is stained with neutral red, has been reported by Becker (37). Furthermore, Nassonov (38) has found that cells placed in an atmosphere of hydrogen contained stained nuclei and colorless cytoplasm and that, when the cells were returned to air, the neutral red left the nucleus and appeared in localized areas in the cytoplasm. Monné (39) stated recently: " The staining of the nucleus has been considered in general as a sign of decreased vitality of the cell leading to its death. In spite of that we find in the literature a few reliable data indicating that vital staining of the nucleus may occur. This has been accomplished by immersing living cells in solutions of various dyes, both of plants and of animals. It has also been accomplished by micro-injecting various dyes, especially the sulphonated pH indicators of Clark and Lubs into amoebae and tissue culture cells. The stain blown against the nuclear membrane is taken up by the nucleus, but after a short time the color disappears. The possibility at least of a transitory vital staining of the nucleus seems to be fairly well established." In his own experiments with amoebae the staining of the nucleus was considered as vital only when the amoeba recovered completely. In all cases of vital nuclear staining, the color faded sooner or later.

As regards colorless cells, in some instances they were considered to be alive (40, 33, 16–27, 5) while in others they were considered to be dead (7, 8, 11, 12). With certain types of tissues the proportion of colorless cells has been small, and consequently many investigators who have employed neutral red as a criterion of viability have disregarded them or have made only passing mention of them. With tissues such as those studied in the present investigation, however, the proportion of colorless cells is high. In such cases it is of importance to know whether unstained cells are alive or dead.

While it is true that cells, when exposed to neutral red, often exhibit localized cytoplasmic staining if alive, and stained nuclei if dead, such distribution of this dye within the cell cannot safely be used as an infallible criterion of viability for the reasons given above. If there are available other criteria of viability the shortcomings of " vital " staining are, of course, not very serious. In studies where other criteria of viability are lacking, however, it would appear that reliance should not be placed upon staining with

neutral red until its applicability to the particular problem in question has first been investigated.

In the light of the more recent work on so-called vital staining, it now seems that staining of cells with dyes like neutral red is not a vital phenomenon but rather a chemical (or physico-chemical) reaction. In many cases this type of staining is closely associated with the living state. In other cases, however, this staining may be retained by cells that have been killed (28). In still other cases, as cited above, living cells may fail to exhibit this phenomenon. In an analogous fashion, nuclear staining is usually associated with cell death, but it has also been observed in cells that were alive (see above). In Nassonov's experiments, changing the environment from hydrogen to air resulted in disappearance of the stain from the nucleus; moreover, there was a concomitant appearance of the neutral red in localized areas in the cytoplasm.

Neutral red, therefore, resembles methylene blue in that the type of staining is dependent not only upon intracellular conditions, but likewise upon the environment of the cell. If, then, not only the diffuse staining with neutral red but also the localized cytoplasmic staining is a chemical reaction, how are the results of staining with this dye to be interpreted? And how are the colorless cells to be classified?

A large number of observers (5, 7, 16–18, 23, 28, 33, 35, 40–47, 52) have noted a correlation between neutral red staining and the physiological condition of the cell. As Ludford (48) has pointed out, the relation between functional activity of the cell and the staining of its cytoplasmic granules and vacuoles has not until recently been the subject of experimental inquiry. Koehring (28) has applied to vital staining the important finding of earlier investigators that neutral red is capable of precipitating proteolytic enzymes without destroying them. She suggested that in vital staining, neutral red is seen in the cell in areas where it is precipitated or concentrated by proteolytic enzymes. The localization of neutral red in digestive vacuoles and immediately around bodies which had been phagocytosed, noted by numerous observers, was considered by Koehring as reenforcing this view. Koehring has interpreted the persistence of neutral red stained bodies in the cytoplasm of heat-killed cells as indicating that the enzymes which had concentrated the neutral red were still intact. This result was obtained only when the cells were killed by heating at comparatively low temperatures for short periods. When the temperature was raised above a critical point, the color disappeared from the granules; Koehring attributed this to destruction of the enzyme by the higher temperature.

If the view that the localization of neutral red is due to combination with certain enzymes should prove to be correct, it might account for the frequent correlation of such staining with the living state. It might also account for the absence of this staining in some living cells as being due to a decreased content of such enzymes. Another possibility is one which involves oxidation-reduction reactions. Clark and Perkins (32), as has been stated previously, have shown that neutral red may be reduced to a leuco form that is readily oxidized back again to the same compound, and that neutral red may also be irreversibly reduced to a yellow fluorescent compound. Rothberger

(49) recorded the formation of a fluorescent material from neutral red in cultures of *B. coli.* In addition, a number of observers have observed the disappearance of neutral red from granules and vacuoles in cells; this may be possibly ascribed to a reduction process.

Solitermann (50) found that monobromacetic acid, which interferes with carbohydrate metabolism and with cell division, also interfered with the staining of cells by neutral red. In tissue cultures which contained a concentration of monobromacetic acid sufficiently high to prevent cell division, the cells failed to show any color whatever in the presence of neutral red. Furthermore, addition of this acid to cells previously stained vitally with neutral red caused decolorization.

It thus appears likely that the staining of cytoplasmic constituents with neutral red is governed by ordinary chemical or physico-chemical processes (54). When the requisite conditions are present in the cell, there are numerous areas which localize the stain; where these conditions are not present, the living cell does not localize the dye.

The results presented in this paper do not require for their interpretation the elaboration of novel concepts. As a matter of fact, thirty-seven years ago Plato (55) carried out interesting experiments with neutral red which led him to conclusions which have bearing on the phenomena described here. He held the views that: localized cytoplasmic staining is not necessarily a vital phenomenon; cells killed quickly may retain the picture of "vital" staining for a short time after death; decolorization may occur in living cells; the staining with neutral red of localized areas in the cytoplasm is dependent upon physiological conditions within the cell; whether a given particle in a living cell does or does not stain with neutral red, depends upon the conditions in that region of the cell where it is situated; neutral red is readily and reversibly reduced; colorless cells and colorless portions of cells may contain neutral red in the leuco form.

What the mechanism is that governs the diffuse staining of the nucleus, or of the entire cell, is still obscure. Cells while in the colorless state in neutral red solutions (and which subsequently stain diffusely) appear, under the conditions of the present study, to be living cells. This is borne out by the striking resemblance of the curves of the colorless spindle cells to the curves of the round cells exhibiting localized cytoplasmic staining. The most satisfactory interpretation of these curves would appear to be as follows:

Round Cells (Fig. 1): At the start, almost all of the cells were alive; only about 10 per cent showed the diffuse staining of dead or moribund cells. As the number of live cells (dot-dash line) decreased, the number of dead cells (continuous line) increased correspondingly. At lower temperatures (Figs. 3 and 5) the same events occurred, but at a slower rate.

Spindle Cells (Fig. 2): At the start, almost all of the cells were alive; only a small proportion showed the diffuse staining of dead cells. As the number of living cells (broken line) [3] decreased, there was a corresponding rise in the number of dead cells (continuous line). At lower temperatures (Figs. 4 and 6) the same events occurred, but at a slower rate. There was thus a

[3] The small proportion of spindle cells that exhibited localized cytoplasmic staining (dot-dash line) were also classified as living.

close parallel between the relative initial proportions of vitally stained round cells and of colorless spindle cells, and a close correspondence between their behavior subsequently.

The curves for the living cells in these experiments parallel in an interesting fashion the transplantation experiments of Sugiura and Benedict (51). These authors immersed tumor fragments in protein-free, isotonic salt solutions for varying lengths of time and tested the transplantability of these fragments. They found that at 37° C. the transplantability fell off rapidly after the first two hours and gave entirely negative results after four hours and six hours, respectively, for the two tumors studied. These transplantation experiments serve as an independent criterion of the viability of tumor tissue in salt solutions.

The loss of transplantability of the tumor fragments in the experiments of Sugiura and Benedict at both body and low temperatures parallels closely the decrease in the number of live cells obtained in the present experiments. In their investigation a decrease of the temperature to 4–5° C. produced a marked retardation of the loss in transplantability. Here, too, the loss of transplantability paralleled the slow decrease in the number of live cells at approximately 8° C. obtained in the present study.

In addition to the cell counts, a few transplantation experiments were performed with minced tissue kept in salt solution in the cold room for a week or more. It was found that sarcoma 37 could be successfully transplanted after immersion at approximately 8° C. for one week, provided that tissue fragments were used for the implants; the preparations that gave positive takes on transplantation contained an appreciable number of living cells, as judged by neutral red staining.

Of course, if implantation of isolated cells into mice would give rise to tumors, the most direct correlation between staining and vitality of tumor cells would be furnished by transplantation of cells selected on the basis of staining. However, this has never been achieved. In this laboratory, isolated tumor cells which were alive as judged by their reaction to neutral red were picked out with a micro-pipette and inoculated into mice; negative results have been obtained to date, even when hundreds of isolated living cells were implanted together.

For certain types of chemical and physico-chemical studies of tumor cells which can best be carried out with micro-manipulative technic, it is of importance to be able to distinguish between living and dead cells at any given time in the experiment. The behavior of tumor cells towards neutral red is capable of furnishing valuable information regarding the vitality of the cells under these conditions, provided that the results are interpreted in the light of the exceptions noted in the foregoing discussion.

Summary

1. Cells of mouse sarcoma 37 were examined with respect to their behavior towards neutral red upon immersion in balanced salt solutions containing this dye. Cells of several normal tissues of the mouse were also examined in the same way.

2. At 38° C., only a small proportion of the cells contained stained nuclei initially; the number of cells with nuclear staining increased with time of immersion. The rate of increase in the proportion of cells with stained nuclei was less the lower the temperature.

3. The two types of cells, designated as "round" and "spindle," preponderating in sarcoma 37 under these conditions differed in their reaction towards neutral red.

4. At 38° C. the great majority of the round cells contained initially stained bodies in the cytoplasm (*i.e.*, vitally stained); the great majority of the spindle cells were initially colorless.

5. Vital staining in spindle cells was observed to fade progressively until the cells were entirely colorless.

6. The subsequent behavior of the vitally stained round cells paralleled that of the colorless spindle cells at all temperatures.

7. Under these conditions, it is probable that the colorless spindle cells are alive.

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