In Vitro Studies of the Metabolism of Tissue Slices

I. Metabolic and Physical Factors Influencing the Penetration of Tetrazolium Salts*

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ABSTRACT

The use of tetrazolium salts for metabolic studies has been dismissed on the basis of their poor penetration into fresh tissue slices. In view of the fact that the penetration of these compounds can be visualized, it was felt that knowledge of the factors involved would be important. Factors, known to influence the penetration of oxygen, were examined with respect to the tetrazolium salts. The penetration of tetrazolium salt into tissue slices was a regular and predictable phenomenon. It was found that decreasing the metabolism of the cells in the slice substantially increased the penetration of these compounds, while increases in metabolism, by addition of substrate (such as succinate) to the incubating medium, considerably decreased their penetration. Increasing concentrations of the salt in the medium resulted in greater but limited penetration. It is our belief that the metabolism of tissue slices can be effectively studied with the aid of the tetrazolium salts, the portion of the population of cells participating in any reaction being accurately established by measuring the depth of the zone of reduced dye.

A classical method for the study of the metabolism of the intact cell under controlled conditions has been the incubation of fresh tissue slices under *in vitro* conditions and the measurement of factors such as the oxygen consumed, the amount of metabolite utilized, or the products formed. Not enough attention has been given to the fact that the validity of these studies depends on the extent to which the slice is penetrated by the variables being measured—the gases, metabolites, or other exogenous agents introduced into the incubating medium. Most experiments dealing with the effects of added substrates or hormones on the metabolism of tissue slices, assume *a priori* that the penetration of these experimental substances

is complete. Quite the contrary is true. Thus, on the basis of theoretical considerations and indirect experimentation, it has been recognized that oxygen penetrates into a tissue slice only to a limited extent (12). Conventional microrespiratory studies must, therefore, remain difficult to interpret since there is no way of determining the completeness of the penetration, and hence the extent to which these substances participate in the reactions observed.

Attempts have also been made to study the metabolism of fresh freehand slices with tetrazolium salts (2, 14, 15). Subsequently this approach was abandoned because of the failure of the tetrazolium salts to penetrate beyond a thin variable zone at the surface of the tissue (3, 10, 11). In view of the many obvious advantages of such an approach, it is unfortunate that no systematic attempt was made to determine the factors which limit the penetration of these salts. It seemed to us that once this aspect of the problem was properly understood, the factor of limited penetration

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TABLE I

Media Used in Incubation Procedures

Stock solutions*	Solution No.		
	1‡	2	3
NaCl (1.8 per cent).............	5.0		5.0
NaCl (0.9 per cent).............	—	10.0	—
KCl (1.15 per cent).............	0.4	0.4	0.4
KH₂PO₄ (2.11 per cent)..........	0.1	0.1	0.1
CaCl₂ (1.22 per cent)............	0.3	0.3	0.3
MgSO₄·7H₂O (3.82 per cent)......	0.1	0.1	0.1
NaHCO₃ (1.3 per cent)..........	0.3	0.3	0.3
PO₄ buffer (0.1 M)..............	0.3	0.3	0.3
Tetrazolium (0.5 per cent).......	5.0	—	5.0
Na succinate (0.1 M)............	—	—	3.0
Methylene blue (0.05 per cent)....	—	0.5	—

* The table indicates parts of stock solution mixed together for the different incubation media utilized.

‡ Basically the same solution utilized by Krebs (7), except that this contains tetrazolium salt.

—rather than being a liability—could serve as a valuable tool for analyzing changes in metabolic activity of tissue slices. The distribution of the reduction product of the tetrazolium salts, being readily visualized, serves as a convenient means of determining directly the number of cells in the slice that have actually participated in the experiment.

Warburg (13) pointed out that the depth to which oxygen penetrates into tissue slices depends not only on physical factors, such as its concentration in the incubating medium and its rate of diffusion through the tissue, but to a large extent on the rate at which it is consumed by the cells in question. It was, therefore, highly suggestive to us to note that there is a close correspondence between the extent to which different tetrazolium salts penetrate tissues and the rates at which they are utilized by biological systems. The salt, 2,3,5 triphenyl tetrazolium chloride (TTC) is reduced more slowly than blue tetrazolium (BT), neotetrazolium (NT), or 2 - p - iodophenyl - 3 - p - nitrophenyl-5-phenyl tetrazolium chloride (INT) (1, 4, 8, 9, 11). The slowest reacting salt, TTC, is the only agent reported to penetrate slices completely (3).

These considerations led us to examine more closely the metabolic factors influencing the penetration of tetrazolium salts into tissue slices. The experiments were designed primarily to determine whether induced changes in metabolism—both

in the direction of increased and decreased activity —had a predictable effect on the deposition of tetrazolium in tissue slices. Consideration was also given to the purely physical elements of slice thickness and the concentration of tetrazolium salt in the incubating medium.

The findings indicate that the penetration of tetrazolium salt into a tissue slice is governed by the same factors shown mathematically by Warburg to be concerned with the utilization of oxygen under *in vitro* conditions. The present experiments also suggest that the tetrazolium technique can profitably be applied to metabolic studies on tissue slices.

Methods

Male rats CFN (Carworth Farms), 175 to 225 grams, were deprived of food for 24 hours prior to experiment. The animals were sacrificed by a blow on the head. The liver, kidneys, and diaphragm were rapidly transferred to iced saline. Freehand slices (0.5 to 1.0 mm. thick) of kidney cortex and liver were prepared with a razor blade. The hemidiaphragms were cut in half. The tissues were washed in saline and then transferred to various incubating solutions shown in Table I. At the end of the incubation periods, the slices were immersed in neutral 10 per cent formalin to halt metabolic activity, cross-sectioned at 15 micra with a freezing microtome, and mounted in glycerin for microscopic examination.

RESULTS

I. Metabolism and Penetration

The initial experiments dealt with the effects of induced changes in the metabolism of tissue slices on the depth of penetration of the tetrazolium salts.

A. Endogenous Reduction of Different Tetrazolium Salts:

Segments of diaphragm, as well as slices of liver and kidney cortex, were transferred to physiological salt solutions (Table I, No. 1) containing either NT or INT, and incubated for 1 hour anaerobically at 37°C. Kidney cortex was also incubated in solutions of TTC and BT.

Kidney cortex (Fig. 1) showed a uniform and quite complete penetration of TTC in slices up to 1 mm. thick, although the crystalline pattern varied with the different areas of the slice, as described previously (3). In contrast, when other salts, NT, BT, or INT were used, the deposits of reduced tetrazolium were restricted to the borders

of the slice (Figs. 4, 7). With these more rapidly reacting salts, the formazan appeared as a dense deposit along the borders of the slices in contrast to the sparse distribution of the TTC crystals. In both liver and diaphragm, the NT and INT like-wise penetrated only to a limited extent.

B. Depletion of Endogenous Metabolites Prior to Incubation in Tetrazolium:

Experiments were then undertaken to determine whether the penetration could be affected by limiting utilization of the salts. Tissue slices were pretreated by incubating them under oxygen and in the presence of methylene blue to intensify further aerobic metabolism. It was felt that such exposure would appreciably deplete the cells of their stores of metabolites (in particular the substrates being oxidized) and by this means decrease the over-all reduction of tetrazolium.

Liver, kidney, and diaphragm were depleted by first incubating the slices in a physiological solution (Table I, No. 2) free of tetrazolium salt, but containing methylene blue. After 4 hours at 37.5°C. in the presence of oxygen, the slices were rinsed in saline and immersed in a standard physiological solution (Table I, No. 1) containing either NT or INT for 1 hour at 37.5°C. anaerobically. Additional studies were conducted with kidney cortex slices incubated in TTC and BT.

The patterns of deposition, in tissues depleted in this way, were distinctly different from those previously observed under control conditions. The cells at the outer borders of the slice showed only negligible deposits of formazan. However, formazan was now found deposited in the more central regions of the slice, showing that the penetration of the tetrazolium salts had been markedly increased. The increased penetration is illustrated by photographs from representative experiments with kidney cortex incubated in each of the tetrazolium salts (Figs. 2, 5, 8).

The size of the depleted area varied with the length of pretreatment. Diaphragm was subjected to the depletion procedure for 1, 2, and 4 hours and subsequently incubated in INT. The margin of cells, which showed loss of ability to reduce tetrazolium salts, was substantially greater after 2 hours (Fig. 11) than after 1 hour (Fig. 10). Diaphragm exposed for 4 hours was completely devoid of any formazan, except for light deposits in the very center of the slice.

TABLE II
Effect of Concentration on Penetration into Kidney Slices

Concentration of tetrazolium salt	Depth of penetration (mm.)			
	NT		TTC	
	Experimental	*Calculated	Experimental	*Calculated
0.1 per cent	0.14	‡	—	—
0.2 per cent	0.19	0.20	0.19	§
0.4 per cent	0.27	0.29	—	—
0.87 per cent	—	—	0.40	0.39

* $d_2 = d_1 (C_2/C_1)^{1/2}$, d_1 is the depth of penetration when the concentration is C_1, and d_2 is the theoretical depth of penetration when the concentration is C_2.

‡ The values for d_2 are calculated from $C_1 = 0.1$ per cent and $d_1 = 0.14$ mm.

§ d_2 is calculated from $C_1 = 0.2$ per cent and $d_1 = 0.19$ mm.

C. Effect of Restoration of Substrate on Tetrazolium Reduction in Depleted Slices:

An obvious next step was to study the restorative action of substrate replacement on penetration. Tissues depleted in the aforementioned manner were subsequently incubated in solutions containing succinate (Table I, No. 3) and tetrazolium salt for 1 hour at 37.5°C. anaerobically. Under these conditions, slices of liver, kidney, and diaphragm now reverted back to the pattern of distribution observed in control experiments.

In the TTC experiments, formazan was present throughout the slice (Fig. 3). A similar reversion to the original pattern was obtained with reconstituted tissues incubated in NT, BT, and INT (Figs. 6, 9, 12). The borders of the slices showed an intense precipitation of formazan which was even more limited than in the control experiments.

II. Effect of Physical Factors on Penetration

A. Concentration of Tetrazolium Salt:

It was anticipated that the penetration into the tissue slice would be influenced physically by the relative concentration of the tetrazolium in the incubating medium. Kidney cortex and liver slices were incubated for 45 minutes aerobically at 37.5°C. in a physiological salt solution (Table I, No. 1) containing either NT or TTC. The experiments were run under aerobic conditions in order to reduce the over-all reduction of tetrazolium and thereby to favor penetration.

By increasing the concentration of tetrazolium salt, it was possible to a limited extent to further its penetration into tissue slices of both liver and kidney (Table II). The calculated values indicated in Table II were obtained by assuming that the penetration of tetrazolium salt was directly proportional to the square root of its concentration in the medium—based on the formula for oxygen utilization developed by Warburg (13). The new depth of penetration, d_2, which should result from a change in concentration from c_1 to c_2, is equal to d_1 (original depth) $\times \sqrt{c_2/c_1}$ The increases in penetration obtained experimentally, closely approximate the values expected from calculation.

B. Slice Thickness:

Inasmuch as the factor of slice thickness was a necessary consideration in the other parts of this investigation, no special experiments were conducted on this aspect of the problem. In designing an experimental procedure, one could select a given slice thickness relative to a given concentration of tetrazolium salt. However, this particular slice thickness could be used only as long as all of the experimental factors were kept constant. The introduction of any factor which would affect the metabolism of the tissue would rule out the basis for using the original slice thickness.

DISCUSSION

The data clearly indicate that the penetration of tetrazolium salts into tissue slices is affected by both physical and metabolic factors. Investigators in the past have given thorough consideration to the penetration of oxygen into tissue slices (5, 6, 12, 13). The relationship of the factors involved in the penetration of oxygen into tissue slices (13) or into a flat sheet of tissue (6) has been expressed by the following formula

$$d = \sqrt{\frac{8DC}{A}}$$

The combined depths (d) to which oxygen penetrates on each side of the slice (or the "limiting thickness" of a slice for such studies) is a function of the rate of diffusion of oxygen through the slice (D), the concentration of oxygen in the incubating medium (C), and the rate at which oxygen is consumed by the cells of the tissue slice (A). Warburg determined that if one changed the gaseous atmosphere above the incubating medium from air

to pure oxygen, the slice thickness for liver could be changed from 0.21 mm. to 0.47 mm. (13). The depth to which oxygen penetrates a tissue slice also depends upon the rate at which it is reduced by the cells. When the rate of oxygen consumption of the tissue is decreased, a greater penetration of oxygen results.

As in the work of Warburg, where an increase in the concentration of oxygen in the medium favored penetration, higher concentrations of tetrazolium salts in the incubating medium definitely increased penetration. By knowing the depth of penetration at one concentration, one can use the formula to determine the depth of penetration d_2 at a different concentration C_2. Such calculations for the tetrazolium salts were in close agreement with experimentally obtained values (Table II).

When the effects of substrate depletion are compared with those of substrate excess, it is clear that utilization of the tetrazolium salt by the cells encountered at the surface, serves to limit its penetration into the deeper portions of the tissue. Following pretreatment designed to deplete tissue activity, little or none of the tetrazolium was utilized by the outer portion of the slice, thereby permitting the salt to penetrate considerably deeper into the slice. Thus, penetration is increased by simply limiting utilization. The presence of zones of heavy formazan deposits within the depths of the depleted slices would seem to indicate that the innermost cells had not been so completely depleted by the pretreatment procedure as marginal cells. It is assumed that when the tetrazolium salts (INT, BT) reached these inner regions, their utilization again was more rapid than their rates of diffusion, resulting in regions of intense deposits beyond which the tetrazolium salts do not penetrate.

Exposure of depleted slices to a substrate, such as sodium succinate, caused a distinctive change in the formazan pattern. With the increased utilization of tetrazolium as a hydrogen acceptor, the pattern reverted to that seen under control conditions where the salt failed to penetrate appreciably into the slice. Shelton and Schneider (11), in an attempt to increase penetration of tetrazolium, were puzzled by the fact that succinate added to the external medium did not increase penetration. This effect is in accord with the concept advanced here that addition of agents, which increase the utilization of substances such as oxygen or tetra-

zolium salt, will further restrict or limit their penetration into the tissue slice.

As utilization of the tetrazolium salt is decreased, the "limiting thickness" or penetration will increase. The present appraisal of the problem demonstrates that even though the deposition is limited, it is a regular and predictable phenomenon. It seems plausible to assume that other substances—including metabolites or hormones—will be affected by the same factors which were found to control the penetration of oxygen and of tetrazolium salts. The tetrazolium salts can serve a unique function by providing a visual means of evaluating the factors involved in tissue slice studies.

On the surface, it would appear that an obvious method of avoiding the physical difficulties imposed by slice thickness would be to prepare slices thin enough to conform with the calculated depth of penetration. Aside from the fact that viable slices thinner than 0.15 mm. would be difficult to prepare without extensive cellular damage, the present study indicates that it is neither necessary nor advisable to resort to attempts to obtain sufficiently thin slices in order to utilize the tetrazolium technique. Activity can be equated not in terms of the misleading total slice weight, as conventionally done, but in terms of the fractional portion of the slice showing deposits of formazan. Formazan can then be extracted with organic solvents and the intensity of color measured in a colorimeter.

Radioautographs prepared from cross-sections of tissue slices incubated with isotope-labelled materials, should prove a useful adjunct for studying the penetration of some compounds.

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EXPLANATION OF PLATES

PLATE 116

Cross-sections of kidney slices incubated in either TTC or NT under various conditions.

FIG. 1. *Control—TTC.* Slice incubated in TTC (solution 1) for 1 hour under a nitrogen atmosphere. Note that formazan is deposited throughout the slice, that the crystals are fine in the center and coalesced clumps in the border, and that the deposits are sparse throughout the slice. × 50.

FIG. 2. *Depleted—TTC.* Slice was first depleted by incubation in the presence of methylene blue (solution 2) under an oxygen atmosphere for 4 hours and subsequently incubated in TTC (solution 1) for 1 hour under a nitrogen atmosphere. A small number of scattered clumps of crystals are seen in the center of the slice with the borders being completely devoid of formazan. × 50.

FIG. 3. *Depleted—Succinate TTC.* Slices were depleted in the same manner as the tissue illustrated in Fig. 2 and subsequently incubated in succinate and TTC (solution 3) for 1 hour under a nitrogen atmosphere. Activity is completely restored to areas devoid of activity in Fig. 2. Deposition of formazan is similar in distribution to that obtained in control slices (Fig. 1), but deposits are not as sparse as in the control slice. × 35.

FIG. 4. *Control—NT.* Deposition of formazan is restricted to the borders of the slice. The intensity of the formazan deposition is much greater than that obtained on reduction of TTC (Fig. 1). × 50.

FIG. 5. *Depleted—NT.* Procedure the same as in TTC experiment for Fig. 2. Deposition is evident throughout the slice, being most dense in the center. Deposition in the borders is greatly diminished in comparison to controls. × 35.

FIG. 6. *Depleted—Succinate NT.* Procedure same as in the TTC experiment for Fig. 3. Intense activity confined to the borders as in the control experiment. (Fig. 4). No evidence of formazan in the center. × 35.
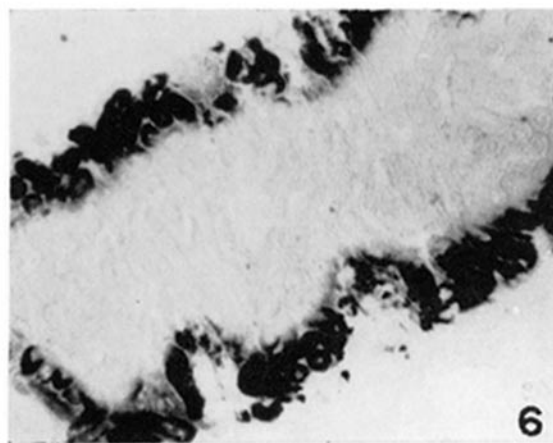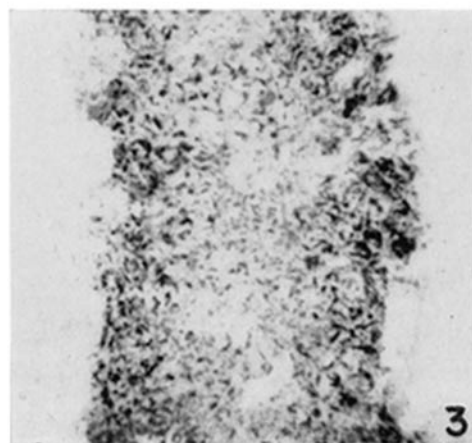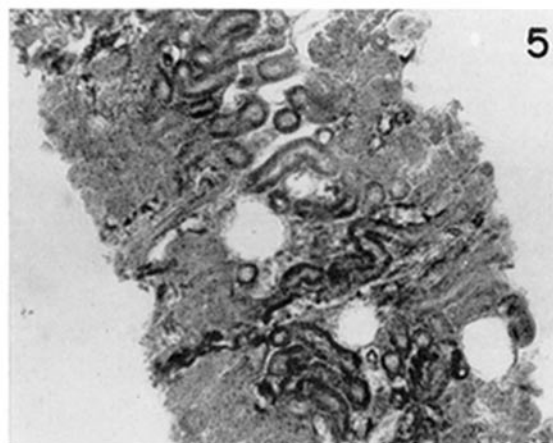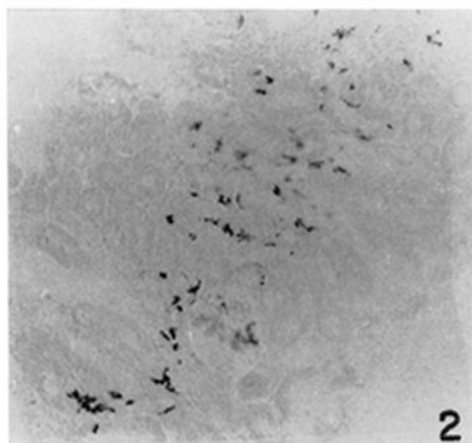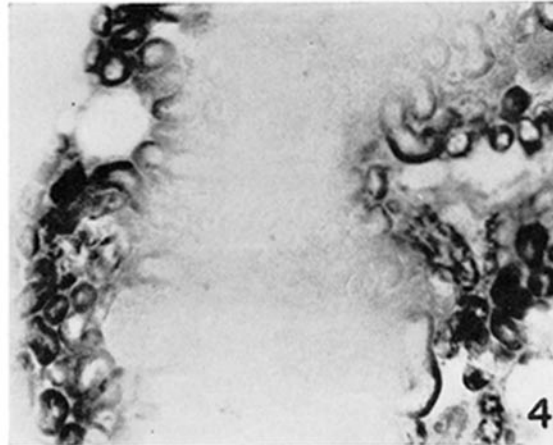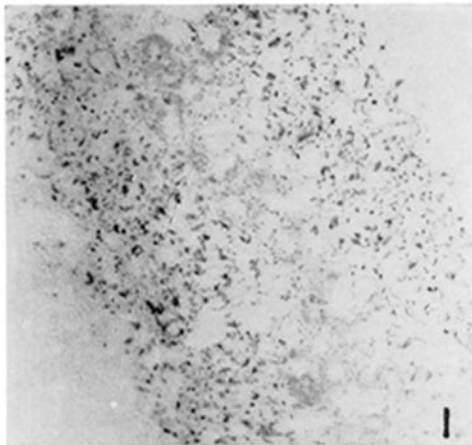
(Cascarano and Zweifach: Metabolism of tissue slices. I)

PLATE 117

Cross-sections of kidney and diaphragm slices incubated in INT under various conditions.

FIG. 7. *Control Kidney—INT*. Deposition of formazan occurs only in the borders of the slice. × 35.

FIG. 8. *Depleted Kidney—INT*. Penetration after depletion is considerably increased and in some areas complete. Note heavier zone of deposition around regions free of crystals. Intensity of formazan deposits in the borders is considerably less than obtained in the control situation (Fig. 7). × 35.

FIG. 9. *Depleted Kidney—Succinate INT*. Addition of succinate to depleted slices restores deposition of INT to the slice borders. Penetration is even more restricted than that obtained in control slices (Fig. 7). × 35.

FIG. 10. *Depleted Diaphragm—INT*. Diaphragm exposed to depletion procedure for only 1 hour prior to exposure to INT. Note that only a thin rim of fibers in the border lose the ability to reduce INT. × 35.

FIG. 11. *Depleted Diaphragm—INT*. Diaphragm exposed to depletion procedure for 2 hours prior to exposure to INT. The number of depleted fibers in the border of the slice has been considerably increased as compared to Fig. 1, by increasing the duration of the depletion procedure. × 35.

FIG. 12. *Depleted Diaphragm—Succinate INT*. Diaphragm exposed for 4 hours to the depletion procedure subsequently incubated in succinate. Note that fibers in the border in Figs. 10 and 11 which did not reduce tetrazolium salt previously, now have a great capacity to do so. × 35.

(Cascarano and Zweifach: Metabolism of tissue slices. I)