Studies on the Reduction of Tetrazolium Salt by Plant Tissues

II. Effect of plasmolysis on the reduction of TTC in plant cell¹

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Received October 4, 1961

It has been known that several physiological activities of plant cells are inhibited by plasmolysis (Kny 1897, Treboux 1903, Walter 1929, Furlinger 1938). But few studies on the effect of plasmolysis were conducted standing on the enzymological point of view. Currier and van der Zweep (1955) have investigated the TTC reduction in leaves of *Anacharis* (*Elodea*) and found that the reaction seems to occur at the ectoplast-cell wall interface and that the amount of formazan produced decreases in a plasmolyzed condition of the cells corresponding to the increase of the molarity of sucrose used as a plasmolyticum. Their studies were conducted only qualitatively. Burström (1958) has studied the effect of hypertonic solution of mannitol on the activity of invertase in roots and coleoptiles of wheat, and found that the enzyme reaction in the root cells decreases rapidly at incipient plasmolysis, while the reaction increases again in hypertonic solutions. Using non-electrolytes as plasmolytica, he suggested that the invertase may locate on the surface of protoplast.

Many cell-physiological investigations have indicated that the protoplast responses in various manners corresponding to different inorganic ions used as plasmolytica. From these phenomena, it has been assumed that the action of ion has intimate relations to the colloidal state of protoplasm. Therefore, it is of interest to investigate the relationship between the extracellular ions added as plasmolytica and the enzymic activities in the plasmolyzed cells.

In a series of papers, the author (Sato 1953, 1955, 1956a, b) has reported that the reduction of TTC by both plant cells and homogenates can be catalysed by succinic dehydrogenase. This reduction of TTC by succinic dehydrogenase was investigated from the standpoint of enzyme chemistry of the cytoplasm, because this reaction is quite sensitive and easy to carry out the quantitative assay of enzyme activity in intact cells.

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¹ Contributions from the Divisions of Genetics and of Cytology, Botanical Institute, Faculty of Science, University of Tokyo, No. 388.

Material and methods

Seeds of *Phaseolus vulgaris* were used as experimental material. This material has been employed already in the previous works on the reduction of TTC by tissue slices and cell-free extracts.

The seeds were immersed in tap water for about three hours in the dark at 25°C. During this period the seeds were soaked and became to be turgid, but showed no indication of germination.

The hypocotyles with radicles (hereinafter these are called as 'embryos' tentatively) were detached from their cotyledons. After the embryos uniformly grown were selected, each embryo was bisected longitudinally, washed with water, and employed in experiment.

The tests of reducing activity of TTC were conducted in Thunberg tubes. The main tube contained 3 ml. of 0.1 M phosphate buffer (pH 8.0), and the bulbed side arm contained 0.5 ml. of 0.1 per cent TTC solution, 0.5 ml. of 0.1 M sodium succinate solution, and 1.0 ml. of pure water. Twenty embryo-slices obtained from ten embryos were placed in the main tube unless otherwise indicated.

To examine the effect of plasmolysis, adequate amounts of plasmolyticum were dissolved in buffer solution in the main tube. When needed, alcohol was added in the side arm in place of pure water.

The Thunberg tubes were evacuated for 10 min. (10 mm Hg) at room temperature and then placed in a 37°C bath for 4 min. before the solutions in the side arms were tipped into main tubes. The tubes were shaken occasionally during the reaction.

The reaction was allowed to proceed 60 min. as usual, and then stopped by replacing the tubes into cold water and opening the stopcocks. The contents, together with slices, were decanted on filter paper. The embryos were collected and removed into 5 ml. glacial acetic-toluene (glacial acetic acid 10 volume, toluene 7 volume). After standing overnight in room temperature, the red formazan was extracted completely by the solvent. The amount of formazan was determined colorimetrically with Hitachi electric photometer using filter No. 47.

The activity was expressed in the optical density of formazan produced in 60 min. by 10 embryos. In an attempt to compare the rate of reduction under various experimental conditions, the relative reaction rate

$$\left(R, \frac{\text{optical density of formazan in test}}{\text{optical density of formazan in control}} \times 100 \right)$$

was used for description.

Results

a. Preliminary experiments

Before the studies on the effect of plasmolysis on the TTC reduction, it

was considered to be necessary to test, whether the quantitative method applied for homogenates is available for tissues with the same accuracy as in the former experiments.

1. *Relation between number of embryos and the rate of TTC reduction*

The number of embryos placed in a tube was varied from 2 to 16, and the reduction was allowed to occur under the same conditions for 60 min. The relation between the number of embryos and the reducing power was investigated by measuring the optical density of the formazan produced in each tube. The results was illustrated in Fig. 1. The activity was propor-

Fig. 1. Relation between number of embryos and optical density of formazan produced.

tional to the number of embryos so far as less than 12 embryos were placed in one tube. But when more than 12 embryos were placed in one tube, the linear relationship could not be observed. In the following experiments, 10 embryos were placed in each tube unless otherwise mentioned.

2. *Time course of the reduction*

In order to test the time course of the reaction, the formazan produced in various reaction time was extracted and measured photometrically. As Fig. 2 shows, the reaction proceeded almost linearly with the time of incubation at least for two hours.

3. *Gas phase*

By the evacuation of Thunberg tubes, not only the oxygen of gas phase was removed, but also the infiltration of the reaction mixture into intercellular spaces were accelerated. Since the reaction by tissue homogenates was strikingly inhibited in an aerobic condition, the following experiments were carried out in an attempt to test, whether or not air does inhibit the reduction in intact cells as in the case of homogenate. The tubes containing the standard reaction mixture and material were evacuated and preincubated as usual. After the temperature equilibrium, the solution in the side arms were tipped into the main tubes, and then the cocks of the tubes were opened to air. Thus the reaction was allowed to occur under aerobic condition. The result is represented by solid circles in Fig. 2. This figure reveals that air does not interfere with the reaction in intact cells. This fact shows a striking contrast to the results by homogenates. But, unless stated otherwise, the following experiments were all conducted under anaerobic condition.

4. Temperature

The temperature during incubation exerts a striking effect on the reduction as shown in Fig. 3. Up to 50°C, the reaction rate increased with temperature. The temperature coefficients were as follows :

°C	Q_{10}
20-30	2.2
30-40	2.4
40-50	2.3

Above 50°C, the activity decreased rapidly, suggesting that the enzyme involved in this reaction is heat labile.

So far as these experiments are concerned, the activity was minimum at 60°C. However, the activity increased again with the temperature over 60°C. Presumably, this is not caused by enzymic reaction, but by sulfhydryl groups of cell protein denatured by heat. Above 50°C, the coloration of tissues was turbid. In the following experiments, the reaction was carried out at 37°C throughout.

Fig. 2. Time course of TTC reduction. ●, anaerobic reduction; ○, aerobic reduction.

5. Effect of pH of surrounding medium

Effect of pH of the surrounding medium on the reduction of TTC by embryo slices was examined over the range of pH 6.2 to 7.8. Fig. 4. shows the relationships between the pH and the activity of embryo slices and homogenates.

In the case of homogenate, the optimum pH appeared at about 7.4, then the curve declined steeply toward both higher and lower pH. In the case of intact cells, however, the activity depended slightly upon the pH of the surrounding medium. The curve showed a gradual declination toward lower pH. Since the reduction of TTC in alkaline medium higher than pH 8 seems to be caused by non-enzymic reaction, the pH of surrounding medium was adjusted to pH 7.8 in the subsequent experiments.

6. Plasmolytic measurement of osmotic pressure of embryo cells

Since the purpose of this study is to test the effect of plasmolysis on

the TTC reduction by intact embryo cells, osmotic values of the cells were estimated by the plasmolysis method. Thin slices of embryo were observed

Fig. 3. Reaction activity as a function of temperature.

Fig. 4. Relation between pH of surrounding medium and optical density of formazan produced.

microscopically in 0.2–0.4 M sucrose solutions, and the concentration of sucrose which caused incipient plasmolysis was regarded as isotonic with the cells. In order to make clear the incipience of plasmolysis, the cells were stained vitally in 1/10000 solution of neutral red before the examination.

It was determined through the repeated observations that the cells of procambium, phloem, and epidermis are plasmolyzed with 0.20–0.25 M and those of pith and cortex with ca. 0.30 M sucrose solution.

b. Main experiments

By adding several kinds of plasmolytica to the buffer solutions, the effect of plasmolysis on the reduction of TTC in intact cells were examined. The final concentration of the plasmolyticum is 3/5 of the initial concentration. The reaction mixture contained not only the plasmolyticum, but also other electrolytes such as buffer salts, substrate and TTC. The final osmotic value of the reaction mixture without plasmolyticum was approximately equivalent to that of about 0.17 M sucrose solution.

In practice, slices were allowed to contact with the plasmolyticum about 15 min. before the start of

the reduction. From the results of the preliminary microscopic observations described above, the time of preincubation was sufficient for the completion of the plasmolysis.

1. *Effect of plasmolysis with sugar solutions*

Effect of sugars on the reduction was tested over the range of 0.06 M to 0.6 M (Fig. 5). Sucrose and maltose showed distinct inhibitory actions even at hypotonic concentrations. The inhibition increased with the concentration of sugars and reached the maximum at 0.3 M. In this concentration, all the cells were perfectly plasmolyzed, and after that, the inhibition did not increase with the concentration. The reason why sucrose and maltose inhibit the reduction of TTC at hypotonic concentrations remains unclear. However, we can not exclude a possibility that sucrose is hydrolysed by invertase, which is known to locate on cell surface, and this hydrolysis may result an increase of molarity of the solution surrounding the cells.

Glucose did not show such a distinct effect on the reaction as the other two disaccharides. The inhibition was not observed unless the concentration became high enough that plasmolysis occurred. The characteristics of glucose which is more permeable to the cells than disaccharide and can act as a substrate for dehydrogenase in

Fig. 5. Effect of sugars.

Fig. 6. Effect of urea.

the cells must be considered.

When the concentration is high enough to complete the plasmolysis in all cells, the plasmolyticum is expected to dehydrate the cytoplasm, and this dehydration must be strengthened with the increase of the concentration. After the activity once reached the minimum level, however, no further decrease of activity was observed. This fact indicates that a mere dehydration of the cytoplasm does not inhibit the reduction of TTC.

2. *Plasmolysis with urea solution*

Applying the same procedure as the case of sugars, the effect of urea on the reduction of TTC was investigated. The result is depicted in Fig. 6 (the curve indicated as 'intact cell'). In contrast to sugars, urea accelerated the TTC reduction markedly in hypertonic concentrations. This phenomenon is considered to be due to an increased permeability of cells caused by the addition of urea.

This seems to be in agreement with the experimental fact that alcohols increased the TTC reduction distinctly at low concentrations (Fig. 7), because alcohols as well as urea are known as effective stimulants to cell permeability.

Fig. 7. Effect of alcohols. Alcohols were added from the side arm together with succinate and TTC solutions. Left, intact cells; right, homogenate.

In the case of tissue homogenate, however, both urea and alcohol inhibited the TTC reduction remarkably (Figs. 6, 7).

3. *Plasmolysis with monovalent electrolytes*

The effect of several inorganic potassium salts on the reduction of TTC

was investigated (Fig. 8). The experimental procedure was the same as that described above. Concerning the plasmolysis induced by electrolytes, the osmotic value depends not only on its molar concentration but also on the van't Hoff's factor (i). From cryoscopical measurements, the reaction mixture containing 0.03-0.08 M mono-valent plasmolyticum was found almost isotonic with the cells of procambium and epidermis. Therefore the cells begin to plasmolyze beyond these concentrations of plasmolytica. In the case of sulphate, 0.05-0.07 M corresponds to the isotonic concentration of the cells.

As it may be seen in Fig. 8, the curves seem to be classified into three groups. The first group is represented by the curves of chloride, nitrate and bromide. These curves are composed of two parts. The activity begins to decrease at the concentration of salts which causes the incipient plasmolysis, and reaches a minimum value at the concentration which causes complete plasmolysis. Then the activity maintained the same level even when the tonicity of the milieu was raised. The effect of sugar seems to coincide with this group. The second group is represented by the curve of KSCN, which shows inhibitory action even at hypotonic concentrations. The inhibition increased rapidly with concentration, and did not reach a constant level. This strong inhibition is probably due to the property of SCN^- ion that can permeate the cells rapidly and causes vigorous swelling of protoplasm. The third group is represented by the curves of K_2SO_4 and KF. Contrary to other electrolytes, these accelerated the reduction of TTC at high concentration. At low concentration, however, a difference between K_2SO_4 and KF was observed. In the case of sulphate, the activity decreased at the concentration which caused the incipient plasmolysis. The initial part of the reaction curve was similar to that of the first group. But in the plasmolyzed tissue, the curve rose again with the increase of tonicity. At the concentrations high enough to complete the plasmolysis, the TTC

Fig. 8. Effect of electrolytes.

reduction was accelerated by sulphate. Although fluoride showed a stimulating effect at all the concentrations, the curve obtained was composed of three parts. In the range of hypotonic solutions, the activity increased with the concentration. However, the activity dropped suddenly at the point of incipient plasmolysis, but maintained a high level above the control. Then the activity increased again at the concentration which causes the plasmolysis, and reached a constant level.

Thus three types of curves were obtained by using several inorganic anions as plasmolytica, but the relation between their concentration and reaction activity are, in general, divided into three steps. The first step was observed, when the salt concentration was below 0.03 M. At this hypotonic concentration, most salts showed no effect on the activity, except fluoride and thiocyanide which are considered to affect the enzyme activity directly. The second step appeared in the range between salt concentration of 0.03 M and 0.08 M, where all had a tendency to inhibit the TTC reduction. This step

seems to correspond to the occurrence of plasmolysis. The third step occurred at the concentrations high enough to induce complete plasmolysis. Under these concentrations, it is characteristic that each salt demonstrated a constant inhibition or acceleration level, independent of its tonicity. Thiocyanide was a single exception.

This general rule also seems to be applicable to the action of plasmolysis with sugars.

Although KSCN, KBr and KF somewhat change the pH of the reaction mixtures, it is clear that differences of the effects of salts may not be caused by change of pH in reaction mixture by the addition of plasmolytica.

In order to compare effects of cations on the reduction of TTC, effects of Na^+ and NH_4^+ were investigated using chloride salts by the same procedure as the case of KCl. The results are given in Fig. 9.

Fig. 9. Effect of chlorides.

No essential difference was observed between Na^+ and NH_4^+ so far as their chlorides were used, although slight differences existed between NaCl , NH_4Cl and KCl . Likewise, effects of Na^+ and K^+ were compared using their nitrates and sulphates as shown in Fig. 10. There was also no difference between Na^+ and K^+ .

4. *Plasmolysis with divalent salts*

In order to examine the effect of cations in more detail, Ca- and Mg-salts were tested as plasmolytica instead of monovalent salts (Fig. 9). In this case, *tris*-buffer was used in place of phosphate buffer, thus the curves of divalent and monovalent salts can not be directly compared. As it may be seen in Fig. 9, both divalent cations demonstrated a strong inhibitory action on the reduction even at very low concentrations, and no essential difference was noted between them. Furthermore, the activity did not decrease even at the concentrations at which the cells are plasmolyzed.

5. *Effect of deplasmolysis*

Embryos were plasmolyzed by immersing in hypertonic sucrose solution (1.0 M) for 60 min, and then deplasmolyzed rapidly by returning to reaction mixtures containing no sucrose, and allowed the reaction to start simultaneously. The experimental condition was the same as previous experiments. The results (Table 1, C) were compared with control series, (A); cells were immersed in buffer solution for 60 min. and then allowed to react in reaction mixture without sucrose, (B); cells were immersed in buffer solution for 60 min. and then plasmolyzed and allowed to react in reaction mixture containing 0.6 M sucrose, (D); cells were plasmolyzed by immersing in buffer solution containing 1.0 M sucrose for 60 min, and then allowed to react in the reaction mixture containing 0.6 M sucrose.

Fig 10. Effect of nitrates and sulphates.

According to Table 1, it is shown that the reduction of TTC in plasmolyzed tissues was, when they were exposed continuously to high osmotic pressure during the reaction (D), inhibited about 17 per cent in their activity ($R_D=83$). This value was almost equal with the value R_B (88). This fact seems to reveal that the duration time of plasmolysis does not affect markedly the activity.

On the contrary, when embryos were plasmolyzed by pretreatment and then deplasmolyzed at the same time with the beginning of the reaction (C), the reduction of TTC was stimulated by about 37 per cent ($R_C=137$). From these data, it is concluded that the deplasmolysis facilitates the reduction. This is resumably due to the increase of the cell permeability by 'plasmolysis-permeability.'

Discussions and conclusions

In the preliminary experiments, the quantitative method for the measurement of TTC reduction by plant tissue was investigated. Although the errors accompanied with the method for tissue or organ as a whole are greater than those for tissue homogenate, the data obtained are reliable practically within a certain limit.

Substances added in the reaction medium affected the reduction rate of TTC by tissue in various degrees corresponding to the kinds of substances and to their concentrations. However, the data seem to permit the following general conclusion that a decrease is observed in the reduction of TTC near the critical concentration of plasmolysis. This conclusion is quite in accord with Burström's data (1958), although the reason why plasmolysis decreases the activity is not clear at present. Concerning the decrease of the activity, Burström has suggested a possibility of partial rupture of protoplast surface, which is considered to be the site of the enzyme. In his interesting review of studies on enzyme activities of cell surface, Rothstein (1954) has stated that, in case of plant cell, several enzymes must be regarded to located on cell surface, *i. e.* between the outer surface of protoplast and cell wall. Currier and van der Zweep (1955) concluded that the plasmolytic inhibition of TTC reduction may be due to disruption of the

Table 1. Effect of pretreatment of slices with or without sucrose

Pretreatment (60 min.)	Buffer solution		Buffer solution with 1.0 M sucrose	
Sucrose in reaction mixture	—	0.6 M	—	0.6 M
R	R_A (100)	R_B 88	R_C 137	R_D 83

structural entities on the surface responsible for the reaction. Although the surface localization of enzymes participating in the intracellular reduction of TTC has not yet been decided through experimental evidences, it may be

reasonable to assume that the enzyme system is located on the protoplast surface and its activity is readily damaged by abrupt separation of wall and protoplast. Such an assumption is supported by the experimental fact that the duration of plasmolysis did not exert any effect on the intensity of the inhibition of TTC reduction (Table 1).

However, the rate of the reduction of TTC reached a constant value, except in the case of KSCN and K_2SO_4 . Above 0.2 M or near this concentration, the reduction rate remained constant even in extremely high concentrations. In Burström's experiment, sucrose inversion increased either after the sudden drop of the activity at the point of incipient plasmolysis or from this point upward, according to the absence or presence of Ca^{++} , and reached later a plateau. These data suggest, that mere dehydration of cytoplasm after its separation from the cell wall does not affect the TTC reduction as well as sucrose inversion.

In Figs. 8, and 9, marked differences appeared among anions in regard to the effect on the reduction of TTC, especially when their concentrations were high enough to induce the complete plasmolysis. Under these high concentrations the order of the relative effectiveness of anions was:

This is the series well known as the lyotropic series or the Hofmeister series for anions. Although the nature of the lyotropic series is still unknown, this order of anions was approximately the same for many other phenomena concerning the colloidal state of protoplasm, *e. g.* viscosity, swelling, gelation and liquifaction, coagulation, and permeability of membrane. Thus the lyotropic series is of fairly general applicability on colloidal phenomena, but not on chemical reactions, so that the anions in our experiments must be regarded to have exerted direct effects not on enzymic reaction itself, but on some colloidal states of protoplasm, resulting in secondary change of formazan formation.

With respect to the strength of the inhibition or the acceleration, no difference was observed among monovalent cations, K^+ , Na^+ , and NH_4^+ , as well as between divalent cations, Ca^{++} , Mg^{++} , but a considerable difference was found between monovalent and divalent cations, especially at low concentrations. In other words, no order of cations was found concerning their effectiveness.

The reduction of TTC by intact cells was different from that by tissue homogenates with respect to the response to some reagents or external conditions. In intact cells, unlike in homogenates, the reduction of TTC was only slightly affected by external pH, and not by aeration. This fact suggests that the activity of the enzyme places at least in part inside the protoplast or in a region where the oxygen concentration and pH can not readily be influenced by the surrounding solution. In addition, the strong acceleration of reduction by addition of urea or alcohols supports the above

assumption, because the reactions which takes place within the permeability barrier must be responsible for effective action of permeability-increasing substances on the reaction. Currier and Day (1954) have reported that formazan production in a yeast cell is accelerated by half saturating the solution with benzene, which may attribute to a more rapid penetration of tetrazolium salt. This consideration is in good agreement with the conclusion mentioned here.

On the contrary, the effect of tonicity on the reduction of TTC suggests a location of the activity in the cell surface. Therefore, it is logical to conclude that the enzymes involved in the reduction of TTC by intact cells is located in part on surface and in part inside the protoplast.

Acknowledgment

The author wishes to thank Professors B. Wada and Y. Ogura for their kind advices and encouragements during the course of this work, and for their revising of the manuscript.

Summary

1. A colorimetric method to determine the activity of TTC reduction in tissue slices of bean embryo was established.

2. The reduction proceeded at constant rate under aerobic condition as well as under anaerobic condition.

3. The reduction rate increased with reaction temperature till 50°C. The pH of surrounding medium influenced the reaction rate slightly.

4. Plasmolysis with sugar solutions depressed the reaction, whereas urea favoured it at high concentration. Deplasmolysis prior to the reaction stimulated the reduction.

5. Plasmolysis with inorganic salt solutions influenced the reduction in different degrees. At the plasmolysing concentrations, anions affected the reaction in the order of the lyotropic series. No serious order was observed among cations.

6. Relationship between the activity of the reaction and site of the enzymes in the cytoplasm and its colloidal state was discussed.

References

- Burström, H. 1958. Influence of plasmolysis and inhibitors on the sucrose inversion in wheat. *Physiologia Plantarum* 11: 771-781.
- Currier, H. B. and Day, B. E. 1954. The tetrazolium reaction in yeast. *Science* 119: 817.
- and van der Zweep, W. 1955. Plasmolysis and the tetrazolium reaction in *Anacharis canadensis*. *Protoplasma* 45: 125-132.
- Fürlinger, H. 1938. Plasmolyse verhindert Ergrünen. *Protoplasma* 30: 328-333.
- Kny, L. 1897. Die Abhängigkeit der Chlorophyllfunktion von den Chromatophoren und vom Cytoplasma. *Ber. D. Bot. Ges.* 15: 388-403.
- Rothstein, A. 1954. The enzymology of the cell surface. *Protoplasmatologia* II-E 4.
- Sato, Si. 1953. The histochemical detection of succinic dehydrogenase with 2,3,5-triphenyl-

- tetrazolium chloride. Bot. Mag. Tokyo 66: 277-285.
- 1955. A supplemental report on the reduction of TTC by plant embryo slices. Bot. Mag. Tokyo 68: 315-319.
  - 1956a. On the reduction of Janus green B by plant embryo slices and mechanism of the specific staining of mitochondria. Bot. Mag. Tokyo 69: 87-90.
  - 1956b. Studies on the participation of succinic dehydrogenase in the reduction of tetrazolium salt by plant homogenates. Bot. Mag. Tokyo 69: 273-280.
- Treboux, O. 1903. Einige stoffliche Einflüsse auf die Kohlensäureassimilation bei submersen Pflanzen. Flora 92: 49-76.
- Walter, H. 1929. Plasmaquellung und Assimilation. Protoplasma 6: 113-156.
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