RELATIONSHIP BETWEEN MAGNESIUM AND THE PHYSIOLOGICAL EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID ON *AZOTOBACTER VINELANDII* AND *RHIZOBIUM MELILOTI*

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The prominent roles that magnesium plays in the economy of the cell are well recognized by workers in the field of bacterial physiology. Its activities range from its effects on cellular division (Webb, 1953) to the many steps in carbohydrate oxidation and the coupled phosphorylation mechanisms. Of the latter the review articles of Brody (1955) and Lehninger (1953-54) have stressed the value of investigations on coupled oxidation-phosphorylation mechanisms to explain many of the fundamental reactions in cellular metabolism. The former author and his co-workers (Brody *et al.*, 1954) have shown with isolated rat liver mitochondria that the tetracycline antibiotics effect uncoupling by complexing essential magnesium. It also has been shown by Brody (1952) that 2,4-dichlorophenoxyacetic acid (2,4-D), the plant growth substance which is extensively used as a herbicide, is an uncoupler of oxidative phosphorylation in rat liver mitochondria.

The vital importance of magnesium to the respiration of azotobacter has been well demonstrated by Goucher and Kocholaty (1954), and the possible dependency of this organism on magnesium for the maintenance and function of its permeability and transport mechanisms has been pointed out by Goucher, Sarachek, and Kocholaty (1955).

A vast amount of experimental work, in both the laboratory and field, has disclosed the manifold diverse responses of plants to the halogenated phenoxy compounds. Although many facets of the action of one of them, 2,4-D, have been investigated and explanations for its mode of action have been suggested, no report has been seen that relates this agent and its mode of action to the bacteria, or higher plants, through the possible involvement of magnesium. This report presents data secured from work with bacteria which may be of aid in elucidating this fundamental biological phenomenon.

MATERIALS AND METHODS

Azobacter vinelandii strain O and *Rhizobium meliloti* Wis. strain 106 were the test organisms. The growth medium for azotobacter was patterned after that of Jensen (1954). It consisted of mannitol, 5 g; calcium glycerophosphate, 0.5 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.006 g; biotin, 0.02 μg ; FeCl_3 , trace; agar, 25 g; and distilled H_2O to 1000 ml. The medium for rhizobium was that recommended by Wilson (1938).

The organisms were grown on agar plates, incubated at 30 C (azotobacter for 24 hr and rhizobium for 48 hr), and were washed from the plates with a solution containing K_2HPO_4 , 2.6 g; KH_2PO_4 , 2.0 g; and NaCl, 3.9 g per L. The harvested cells were washed twice with the above solution by centrifugation at 3,000 rpm and 10 C for 45 to 60 min; were resuspended in the salt solution and then filtered through Reeve Angel filter paper no. 202. To standardize the quantity of cells added to each Warburg flask, they were diluted with the salt solution to give an optical density reading of 0.4 when determined with a B & L Spectronic 20 spectrophotometer at 420 m μ ; one ml of this standard suspension was used in the tests.

A different procedure was used for obtaining the *A. vinelandii* cells for tests on the phosphate effect (Johnson, Magee, and Colmer, 1956). These cells were grown in Burk's mineral salt medium (Wilson and Knight, 1952) with mannitol as the carbon source in 300 ml amounts in 1000-ml flasks. The flasks were agitated on a rotary shaker for 16 to 18 hr. The cells were centrifuged at 3,000 rpm and 4 C for 10 min and then washed once in 0.15 M KCl. They subsequently were filtered and diluted with 0.15 M KCl to obtain the same optical density reading as described for the other cells.

Triple distilled water was used for the media and all solutions. The triethanolamine salt of

2,4-D, 40 per cent acid equivalent, was secured from the Dow Chemical Company. Oxygen uptake was measured manometrically in a conventional Warburg apparatus.

RESULTS

Relation between magnesium and the inhibition by 2,4-D of oxygen uptake of A. vinelandii. Figure 1 shows that without added magnesium the normal oxidative activity of the azotobacter (curve 1) proceeds at the usual high rate characteristic of these organisms. This oxidative activ-

Figure 1. Relation between magnesium and inhibition by 2,4-D of oxygen uptake of *Azotobacter vinelandii*. Components given as final concentration in 2.5 ml. Main compartment: phosphate buffer, 0.03 M, pH 7.0; mannitol, 0.008 M; MgSO_4 , 0.0032 M in flasks 2 and 4; cell suspension, 1 ml. Side arm: 0.2 ml of 2,4-D to give final concentration as indicated on graph. Center well: 0.1 ml of 10 per cent KOH with paper strip. Gas was air; temperature was 31 C.

Figure 2. Relation between magnesium and inhibition by 2,4-D of oxygen uptake of *Rhizobium meliloti*. Components given as final concentration in 2.5. Main compartment: phosphate buffer, 0.03 M, pH 7.0; glucose, 0.0072 M; MgSO_4 , 0.0032 M in flask 2 at 0 hr and 0.0016 M in flasks 1 and 3 after T_2 ; 2,4-D, 0.0185 M after T_1 and 0.037 M after T_2 in flask 2 and 0.0185 M after T_1 in flask 3; cell suspension 1 ml. Center well: 0.1 ml of 10 per cent KOH with paper strip. Gas was air; temperature was 31 C.

ity is almost completely inhibited by 1.85×10^{-2} M 2,4-D at this same low level of magnesium (curve 3). A marked protection from this inhibition by 2,4-D is afforded the organism by the prior addition of 0.2 ml of 0.04 M $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ to the assay system at zero time (curve 2). In the presence of this added magnesium, 3.7×10^{-2} M 2,4-D (twice the concentration of the agent as used in curve 3) is required to inhibit completely the oxidative degradation of mannitol by the organisms (curve 4).

Relation between magnesium and the inhibition by 2,4-D of oxygen uptake of R. meliloti. The phenomenon has been extended to a system involving the oxidative dissimilation of glucose by *R. meliloti* (figure 2). Curve 2 demonstrates that the presence of added magnesium at zero time followed by 2,4-D at T_1 protects the organism, as was observed with *A. vinelandii* (note curve 2, figure 1). The second application of 2,4-D at T_2 , however, inhibits the oxygen uptake of *R. meliloti* completely.

Curve 3 shows that when added magnesium is lacking at zero time, the inhibition produced by

2,4-D at T_1 was not reversed by the later addition of magnesium at T_2 . It should be noted here that the apparent increase in inhibition brought about by the addition of magnesium at T_2 (curve 3) is not real. In other experiments it was evident that a period of time, represented here by the 15 min period between the point of addition of the 2,4-D and the next reading, was required in the case of *R. meliloti* before the full effects of the added 2,4-D became manifest upon the organisms. Thus, that portion of the curve shown after the tipping at T_2 illustrates that the inhibition is a continuing function of the 2,4-D added and that the addition of magnesium at T_2 neither increases nor decreases the inhibition.

Relation of phosphate to the enhanced toxicity of 2,4-D. The presence of phosphate has been reported to increase the inhibitory action of 2,4-D on nitrate uptake by excised wheat roots (Nance, 1949), and on oxygen uptake by *A. vinelandii* (Johnson, Magee, and Colmer, 1956). Table 1 shows that with a minimal amount of magnesium this organism respire quite actively, and with added magnesium (to give a final 0.003 M concentration) there was some stimulation in its oxygen uptake. When 2,4-D was added to these two systems, there was a marked difference in the oxygen uptake; with added magnesium in the system, almost complete protection was afforded the organism from the deleterious action of 2,4-D. These results confirm the findings of the tests illustrated in figures 1 and 2 and serve as a control background for the tests with phosphate.

Phosphate added (50 μ moles) to either the control system with minimal magnesium or to the control system with added magnesium produced no change in the oxygen uptake by the organism. However, when 1.85×10^{-2} M 2,4-D was added to these two phosphate and magnesium systems, that system with the minimal concentration of magnesium was markedly altered; very little respiratory activity was evident by the end of the 30 min period, yet the system with the added magnesium was again afforded protection by the increased magnesium content.

By means of a procedure similar to that of Brody *et al.* (1954), a relationship between these three components may be demonstrated without the presence of the test organisms. When 227 μ moles of 2,4-D and 32 μ moles of $MgSO_4 \cdot 7 H_2O$ are diluted to 10 ml with 0.25 M phosphate

TABLE 1

The relation of phosphate and magnesium to the toxicity of 2,4-D for Azotobacter vinelandii

Systems	O ₂ Uptake	
	15 min	30 min
	μL	μL
Endogenous	0	0
Active respiration (minimal Mg ⁺⁺)	51	114
Active respiration (added Mg ⁺⁺)	75	162
Minimal Mg ⁺⁺ and 1.85×10^{-2} M 2,4-D	58	78
Added Mg ⁺⁺ and 1.85×10^{-2} M 2,4-D	78	148
Minimal Mg ⁺⁺ and 50 μ moles PO ₄ ⁼	62	127
Added Mg ⁺⁺ and 50 μ moles PO ₄ ⁼	73	159
Minimal Mg ⁺⁺ and 1.85×10^{-2} M 2,4-D and 50 μ moles PO ₄ ⁼	60	72
Added Mg ⁺⁺ and 1.85×10^{-2} M 2,4-D and 50 μ moles PO ₄ ⁼	66	116

Each Warburg vessel contained in a total of 2.5 ml a final concentration of 0.03 M phosphate buffer, 0.008 M mannitol, 0.0032 M $MgSO_4$ (where indicated) and 1.0 ml cells. $MgSO_4$ was added at 0 hr; 2,4-D or phosphate or 2,4-D and phosphate were added at 15 min.

buffer, pH 7, and the mixture allowed to react overnight at 30 C, a precipitate forms. It is possible that this may parallel what occurs in physiological systems where these three substances are involved.

DISCUSSION

It is evident from the results presented that the inhibition of the oxidative activity of the two bacteria by 2,4-D is directly related to the concentration of available magnesium, and two possible explanations emerge. In the first place, the chemical structure of 2,4-D makes it seem possible that it may be a chelating agent for divalent cations. Thus, its complexing with magnesium could deplete a physiological system of essential magnesium and thereby impair oxidation, phosphorylation and those other mechanisms, catabolic or anabolic, which are dependent upon specific concentrations of available magnesium.

A second possible explanation for these data is that the toxicity of 2,4-D might be by some

other critical mechanism and in the experiments reported here the complexing with magnesium prevented its acting in this other way. But the fact remains that if 2,4-D by complexing with magnesium is prevented from performing some other inhibitory activity of which it is capable, this is at the simultaneous expense of the magnesium of the system, an expense which is quite costly in the physiological economy of the cell. It is interesting to note in this regard the emphasis that Brody (1955) placed upon the complexing of essential magnesium by the tetracycline antibiotics in his explanation for the action of these agents upon his test system.

The establishment of the exact degree of inhibition of a given mechanism known to be located in the interior of the cell by a given concentration of 2,4-D will carry significance only when it is established to what degree, if any, 2,4-D penetrates the cell. However, whether or not 2,4-D gains entrance into the cell, its complexing of magnesium in the external environment or at the cell surface will affect the magnesium-dependent activities at the cell surface. Any loss of magnesium in the external environment could also interfere with the maintenance and function of permeability and transport mechanisms (Goucher, Sarachek, and Kocholaty, 1955).

At present the first hypothesis is favored, but, in either case, it is felt that the phenomenon revealed here helps to explain the results secured over the past 4 years with 2,4-D and its components upon bacteria (Johnson and Colmer, 1955*a*, *b*; Magee and Colmer, 1955, 1956; and Johnson, Magee, and Colmer, 1956).

The great role that 2,4-D and allied chemicals are playing in agriculture makes it quite fascinating to speculate that its mode of activity on the higher plants might be elucidated by the findings gained from a study of it with bacteria. And further, if the core mechanism of the action of this halogenated phenoxyacetate is directly related to the magnesium-dependency of the metabolic processes of the organism, and if phosphate can enhance the toxicity of the 2,4-D, then it might be of great import to consider the use of such additives to increase the toxic action of this herbicide to those plants which seem to be rather insensitive to presently used rates of field application.

SUMMARY

The inhibition by 2,4-Dichlorophenoxyacetic acid (2,4-D) of oxygen uptake of *Azotobacter vinelandii* strain O and *Rhizobium meliloti* Wis. strain 106 has been shown to be intimately associated with magnesium.

Marked inhibition of oxygen uptake was produced by 1.85×10^{-2} M 2,4-D in media with minimal amounts of magnesium. Protection from this inhibition was afforded the organism by the prior addition of magnesium in a final concentration of 0.003 M $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$. At this level of magnesium, 3.7×10^{-2} M 2,4-D was required to effect complete inhibition of oxygen uptake.

The simultaneous addition of phosphate with 2,4-D enhances the toxicity of the latter to *A. vinelandii*. This enhanced toxicity is related to the magnesium concentration of the environment.

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