*Short Communication*

# A Technique for the Assay of Enzymes in Intact Plant Cells in the Presence of Dimethylsulfoxide[1]

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In our studies on the biosynthesis of tryptophan in callus tissue of *Nicotiana tabacum* var. Wisconsin 38 (3, 4), we have made use of a convenient technique for the assay of enzymes in intact cells of this tissue. The technique involves the use of dimethylsulfoxide (DMSO), an agent which appears to alter the permeability properties of many cell types without effecting the activity of a variety of enzymes (1). By altering the permeability of intact cells with DMSO in order to facilitate the entry of substrates and release of reaction products, we hoped to observe activity for the enzymes of the tryptophan pathway without initially encountering the technical problems of denaturation which often occur in plant tissues in the preparation of cell-free extracts. Because our most extensive studies have been done, both in whole cells and in partially-purified preparations, with the terminal enzyme, tryptophan synthetase, data for this enzyme are presented here in order to show some of the characteristics of the whole-cell assay system.

The tryptophan synthetases of microbial origin all catalyze 3 reactions (2, 6): 1) the conversion of indoleglycerol-P and L-serine to L-tryptophan and glyceraldehyde 3-P, 2) the condensation of indole and L-serine to form L-tryptophan, and 3) the reversible reaction involving the cleavage of indoleglycerol-P to indole and glyceraldehyde 3-P. Reactions 1 and 2 require pyridoxal phosphate as a cofactor; these reactions have been demonstrated for the *N. tabacum* tryptophan synthetase both with assays of intact cells and of partially-purified preparations. Reaction 3 has not been demonstrated under any conditions. All data presented here were obtained with the reaction 2 assay; the production of $^{14}$C-L-tryptophan from the substrate $^{14}$C-indole was measured. One exception is the datum for the K*m* for indoleglycerol-P, where $^{3}$H-indoleglycerol-P was substituted for indole as substrate and reaction 1 was measured. Methods for the preparation and assay of the partially-purified enzyme are presented elsewhere (4).

Procedures for growth of *N. tabacum* callus tissue have been described elsewhere (3, 4). For a standard assay, 1 g of friable callus tissue was taken directly from agar medium and placed into a 3 ml reaction mix containing 600 μmoles potassium phosphate (pH 8.5), 120 μg pyridoxal phosphate, 120 μmoles L-serine, 0.6 μmole of 2-$^{14}$C-indole (specific radioactivity 350 cpm per mμmole), and DMSO to 10 % v/v. (The DMSO was obtained from J. T. Baker Chemical Corporation). The reactions were incubated at 37° and terminated by placing the tubes in a boiling water bath for 5 min. The cells were removed by centrifugation and the supernatant assayed for the reaction product, L-tryptophan, as described elsewhere (4).

Table I. *The Effect of DMSO on the Entry of Substrates and Release of Reaction Product*

The reaction was terminated after 1 hr by the methods described in column 2. The supernatants were then analyzed for $^{14}$C-L-tryptophan.

| DMSO at 10 % in reaction | Method of recovery of tryptophan from cells | mμmoles $^{14}$C-tryptophan released per g cells per hr |
|---|---|---|
| Absent | Cells boiled and centrifuged | 8.5 |
| Absent | Cells centrifuged only | 2.1 |
| Present | Cells boiled and centrifuged | 11.4 |
| Present | Cells centrifuged only | 10.4 |

The data of figure 1 and table I have been presented in order to show some of the effects of DMSO on the assay system. It was initially observed that the reaction was not completely serine-dependent, due presumably to the presence of a small endogenous pool of serine in the intact cells. In order to demonstrate complete serine dependence, cells were pre-incubated for 15 min at 37° on a

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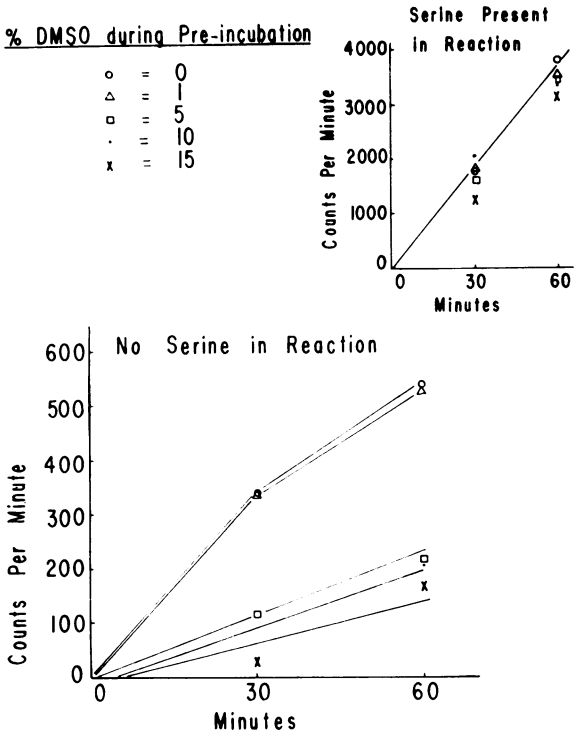


FIG. 1. The leaching of serine pools in the presence of DMSO. Counts per min equals the amount of $^{14}$C-L-tryptophan produced from $^{14}$C-indole in reaction 2. Cells were preincubated 15 min in 0.1 M potassium phosphate (pH 8.0) plus DMSO at the concentrations indicated. Cells were then collected by filtration and assayed in reaction mixes containing 15 % DMSO, buffer, $^{14}$C-indole, and pyridoxal phosphate, but no serine. The inset shows activity from identically-treated cells when serine was present in the reaction.

rotary shaker in 0.1 M potassium phosphate (pH 8.0) plus varying concentrations of DMSO. The cells were collected by filtration and assayed in the presence and absence of serine in reaction mixes containing 15 % DMSO. The results of such an experiment are shown in figure 1. The data show that pre-incubation in buffer plus 1 % DMSO left the characteristic serine dependence of that obtained when cells were pre-incubated in buffer alone. Pre-incubation in 5, 10, or 15 % DMSO caused a significant reduction in tryptophan synthetase activity assayed in the absence of added serine. Presumably then, at these higher concentrations, DMSO caused a leaching of the normal endogenous serine pool. It has been subsequently shown that further incubation in 10 % DMSO can leach out enough of the serine pool to make the reaction completely serine dependent. In addition, the inset to figure 1 shows that when serine was added back to cells from the various pre-incubation conditions, the reaction rates were nearly identical, except for a slight lowering of activity in the cells pre-incubated in 15 % DMSO. On the basis of experiments such as these, 10 % DMSO was chosen as a routine concentration for the assay.

The data of table I further describe the effects of DMSO on the assay system. In this experiment, assays were performed in the presence and absence of 10 % DMSO. In both cases the reaction was terminated in 2 different ways—by placing the assay tubes in a boiling water bath for 5 min and then centrifuging out the cells, or simply by centrifuging the cells with no heat treatment. The supernatants were then analyzed for tryptophan. It can be seen that in the presence of 10 % DMSO at least 90 % of the tryptophan formed during the incubation was excreted into the supernatant relative to cells whose tryptophan was released by disruption of the cells by heat treatment. In the absence of DMSO, 75 % as much tryptophan is produced per hr, but only one-fourth of this was excreted by the cells, and the kinetics of release showed that release only began to occur after 30 min incubation at 37°. These results have been interpreted as showing that in the absence of DMSO, the rate of entry of substrates used in this assay is not nearly so much retarded as is the release of newly-synthesized tryptophan relative to the situation in the presence of 10 % DMSO.

The usefulness of the whole-cell assay for determining some of the properties of the enzyme is illustrated in table II which compares data obtained in the whole-cell assay to those obtained with a partially-purified preparation of the enzyme. The data of table II show that an identical pH optimum (the shape of the curve is also identical) as well as

Table II. *Comparison of Values for Enzymatic Properties of* N. tabacum *Tryptophan Synthetase Assayed in Intact Cells and Partially-purified Preparations*

Prior to assaying whole cells, the cells were preincubated 30 min in buffer plus 10 % DMSO to remove endogenous substrate pools. Methods for preparation and assay of the partially-purified enzyme are presented elsewhere (2). *Km's* were determined by the method of Lineweaver and Burk (5).

| Property measured | Whole-cell assay | Assay of partially-purified preparation |
|---|---|---|
| pH Optimum | 8.2 | 8.2 |
| *Km*, Indole | 0.027 mM | 0.016 mM |
| *Km*, Indoleglycerol phosphate | 0.23 mM | 0.11 mM |
| *Km*, L-Serine | 0.72 mM | 34.0 mM |
| *Km*, Pyridoxal phosphate | N.D.[1] | 1.3 μM |

[1] Not determined.

similar K*m* values for the substrates indoleglycerol phosphate and indole were obtained with the 2 different assays. The K*m* value for serine was markedly higher for the partially-purified enzyme; the reason for the difference is not known, but it may be due to the presence of another enzyme which competes for serine which was concentrated and/or activated in the process of partial purification, or to a change in the tryptophan synthetase itself upon isolation. A complete dependence on the cofactor pyridoxal phosphate has not been demonstrated in the whole-cell assay even after extensive pre-incubation of cells in DMSO, although the reaction can be stimulated some 30 % by the addition of pyridoxal phosphate. This effect could well be due to the high affinity of the enzyme for this molecule which would make complete removal by the pre-incubation process more difficult.

A study of the effect of DMSO on the partially-purified enzyme has shown that the reaction 2 activity is fully active in the presence of 10 % DMSO, but shows a slight inhibition (about 30 %) in the presence of 15 % DMSO. On the other hand, reaction 1 activity is rather strongly inhibited (about 80 %) in the presence of 10 % DMSO, although the inhibition apparently does not involve a significant change in the binding of indoleglycerol phosphate to the enzyme (see table II, K*m* for indoleglycerol phosphate).

We have successfully used the whole-cell assay for the demonstration in *N. tabacum* callus tissue of 4 of the enzymes specific to the pathway of tryptophan biosynthesis, as well as an unrelated enzyme, an apparent β-galactosidase, as defined by its ability to split *o*-nitrophenyl-β-D-galactoside to *o*-nitrophenol. Thus, the technique seems suitable for a wide variety of assays with this tissue. We have also found tryptophan synthetase activity with the whole-cell assay in callus tissue of *Happlopappus gracilus,* and in callus tissue from another strain of tobacco, but we have no further information as the usefulness of the assay with other plant tissues. However, the use of DMSO for other purposes on such a wide variety of cell types, including cells of animal, plant, and bacterial origin, suggests that such an assay may be applicable to a number of different cell types.

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