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# Enzymic Conversion of Reticuline to Salutaridine by Cell-Free Systems from *Papaver somniferum*†

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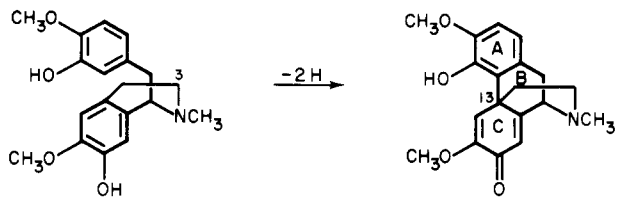
ABSTRACT: A cell-free extract from the opium poppy, *Papaver somniferum*, was prepared that utilized hydrogen peroxide to convert (±)-[3-$^3$H]reticuline to [$^3$H]salutaridine in 80–85% yield based on consumed [3-$^3$H]reticuline. The phenolic-coupling enzyme activity was not detected in the crude homogenate of whole plants. Reticuline conversion to salutaridine was accomplished only after methods were developed to separate both alkaloid and hydrogen peroxide degrading activities from the desired enzyme activity by centrifugal fractionation of carefully prepared stem and root extracts. Purity of the enzymatically produced [$^3$H]salutaridine was established by chromatographic methods and synthetic conversion to [$^3$H]-thebaine.

Biosynthesis of the morphinan alkaloids in the opium poppy has been shown by tracer studies to involve the intramolecular coupling of reticuline (**1**) to yield salutaridine (**2**) (Barton et




1 2

al., 1965). Bond formation occurs between the hindered ortho position of one aromatic ring and the substituted para site of the other, resulting in oxidative coupling and loss of aromaticity in the latter, ring C.

The biochemical stereoselectivity of the reaction has no equivalent in synthetic organic chemistry. Extensive efforts to develop chemical conditions for this critical step in the biomimetic synthesis of morphine have given low-yield conversions complicated with isomeric side products (Szantay et al., 1980). This situation has been somewhat alleviated by the use of a symmetrical ring A precursor, with the accompanying burden of removing the symmetrizing additional aromatic hydroxyl group (Schwartz & Zoda, 1981).

Isolation of enzymes converting reticuline to salutaridine would allow the study of both this novel biochemical system and the unusual chemistry under its control. However, previously reported attempts to obtain even a crude preparation containing this activity have had little success. The extracts obtained from poppy latex by centrifugation converted labeled 3,4-dihydroxy-L-phenylalanine into morphine (0.05% incorporation), thus implying a conversion of reticuline to salutaridine (Fairbairn & Steele, 1981). However, other studies on latex fractions failed to detect oxidation of added reticuline (Roberts, 1971).

Enzyme extracts of rat liver converted reticuline into non-morphinan products (Kametani et al., 1980). In cell cultures of opium poppy, (−)-reticuline was selectively inert to metabolism and no transformation to salutaridine or morphinan alkaloids was detected (Furuya et al., 1978).

Limited conversion of (−)-reticuline to (+)-salutaridine, the precursor of the pharmacologically active opiates, has been achieved in fermentation cultures of certain microorganisms (Schoenewaldt & Ihnen, 1974). However, the yield of salutaridine was poor, ranging from 1 to 10%. In contrast, introduction of either (+)- or (−)-reticuline into whole plants results in good incorporation into morphine, indicating conversion of both reticuline isomers to (+)-salutaridine (Battersby et al., 1965; Borkowski et al., 1978).

The immediate obstacle to investigating enzymatic conversion of reticuline to salutaridine has been the lack of even a crude active enzyme preparation. Our previous studies on an enzyme extract that reduced codeinone to codeine suggested methodology that we have adapted to the study reported here (Hodges & Rapoport, 1980). As before, whole poppy plants seemed a better initial source for the alkaloid-synthesizing enzyme system than latex, plant cell cultures, or microbes. Only in plants has the natural occurrence and the conversion of reticuline to (+)-salutaridine been established. As in the codeinone reductase studies, so that high sensitivity and reaction specificity could be achieved, [3-$^3$H]reticuline was em-

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ployed as the probe for the sought after enzymic conversion.

## Experimental Procedures

### *Materials*

[3-$^3$H]Reticuline and unlabeled reticuline were synthesized by reported procedures (Borkowski et al., 1978). These materials were further purified by preparative thin-layer chromatography (PTLC) ($SiO_2$, 1 mm thick; $CHCl_3$/MeOH/$Et_3N$, 80/20/1) and high-performance liquid chromatography (HPLC) (Altex $SiO_2$ column, 5-$\mu$m particles; 10 mm i.d. × 250 mm; $CHCl_3$/MeOH/$Et_3N$, 80/20/0.1; flow rate 2.0 mL/min; detector at 280 mn; retention time 13.0 min). The specific activity of the purified [3-$^3$H]reticuline was 7.96 × $10^6$ dpm/$\mu$mol.

Salutaridine was synthesized by bisulfite conversion of thebaine to demethylsalutaridine (Bjeldanes & Rapoport, 1972), followed by methylation with diazomethane (Rearick & Gates, 1970). The salutaridine was purified by PTLC ($SiO_2$, 1 mm thick; $CHCl_3$/MeOH/$Et_3N$, 90/10/1) and HPLC (Altex Ultrasorb ODS column, 10 mm i.d. × 250 mm; MeOH/$H_2O$/$NH_4OH$, 80/20/0.1; flow rate 1.5 mL/min; detector at 243 nm; retention time 17 min).

The enzyme extracts were prepared from bolting, preflowering specimens of *Papaver somniferum*, the origin of which has been previously detailed (Hodges et al., 1977). Plant ages ranged from 50 to 85 days.

### *Methods*

*Preparation of Extracts.* The plants were washed and the fine roots were removed. The rest of the plants was used in the initial whole plant studies. In later experiments the stems, roots, and midribs of the larger leaves were used.

Extracts were prepared from the weighed fresh plant material in a nitrogen-purged glove box in a cold room (3 °C). The plants were cut into 1-cm pieces, the pieces being allowed to fall directly into buffer previously deoxygenated by bubbling nitrogen. Generally the volume/weight ratio of buffer to plant material was 2/1. Depending on the experiment, the plants were either ground in bursts of several seconds in a glass blender or mashed in a mortar with a pestle. In a few experiments, the pieces without buffer were frozen with liquid nitrogen, ground into a powder, and thawed as buffer was added. Each homogenate was filtered through Miracloth to give a crude extract. In the first experiments the extract was assayed directly.

In later experiments, the crude extract was placed in a gas-tight centrifuge tube, removed from the $N_2$ box, and centrifuged at a low speed to give the low-spin pellet. The tube was reentered into the $N_2$ box and the supernatant transferred to a new tube for high-speed centrifugation. The final high-spin pellet and supernatant were obtained in this manner. In particular cases, the pellets were washed by resuspension in fresh buffer under a nitrogen atmosphere and centrifuged to re-form the pellet. The enzyme extract was prepared from the pellet simply by resuspension in the appropriate buffer.

*Enzymatic Conversion of [3-$^3$H]Reticuline to [$^3$H]Salutaridine.* In the $N_2$ box, a 3-mL portion of the enzyme extract was placed in a septum-capped vial. The closed vial was removed from the box and placed in a water bath at 24 °C for 2–3 min. Into the vial was injected by syringe various solutions appropriate for the particular assay: 50 $\mu$L of 2% $H_2O_2$; 50 $\mu$L of mixed cofactors [nicotinamide adenine dinucleotide (NAD), 0.5 mM; adenosine 5′-triphosphate (ATP), 10 mM; $MgCl_2$, 10 mM; *S*-acetyl coenzyme A sodium salt (CoA), 0.5 mM; reduced glutathione, 1.0 mM]. After the mixture was swirled, 50 $\mu$L of 1 mM AcOH containing approximately 3 $\mu$g of [3-$^3$H]reticuline was injected. The amount of [3-$^3$H]reticuline was determined exactly by scintillation analysis of an aliquot of each solution. In assays testing oxygen as a reaction component, the enzyme extract and desired cofactors were added to an assay vial by using the above inert-atmosphere methodology. The vial was then opened and $O_2$ gas layered over the solution. Rapidly, the vial was recapped and an aliquot of [3-$^3$H]reticuline in solution was injected.

The assay vial was transferred from the water bath to a wrist-action shaker and gently rocked, avoiding foam production. The assay mixtures were incubated for 15 min, except in the time course experiments. After the incubation, the enzymic reaction was stopped by adding 50 $\mu$L of 85% $H_3PO_4$ and cooling the vial in ice–$H_2O$. To the assay mixture was added 300 $\mu$L of MeOH containing 150 $\mu$g each of nonradioactive reticuline and salutaridine, the solution was extracted twice with 2 mL of $CHCl_3$, $CHCl_3$ and insolubles were discarded, and to the aqueous phase was added $NH_4OH$ to pH 8.3–8.9. The solution was extracted twice with 3 mL of $CHCl_3$/propanol (3/1), and the organic phases were removed, combined, and evaporated under $N_2$, with traces of $H_2O$ removed under vacuum. The residue was resuspended in 75–100 $\mu$L of $CHCl_3$/MeOH (2/1) and spotted on a TLC plate ($SiO_2$, 250 $\mu$m thick, with fluor), which was developed in $CHCl_3$/MeOH/$Et_3N$, 90/10/2. The salutaridine and reticuline bands were removed separately and the alkaloids freed from silica by elution with $CH_2Cl_2$MeOH, 4/1. The organic solvents were evaporated under $N_2$ and the alkaloids redissolved in MeOH. Analysis for mass was based on UV absorption, for salutaridine at 243 nm and for reticuline at 280 nm. For total elimination of interference by traces of the TLC fluor, reticuline analysis was also performed by quantitative HPLC under the same conditions listed for purification. Portions of the methanolic alkaloid solutions were used for radioactivity analysis by standard liquid scintillation methods and are precise to ±5% for each analysis. Protein analyses were performed by the method of Lowry et al. (1951), using BSA standards.

*pH-Dependence Experiments.* Assay solutions were prepared from the fractions so as to avoid local pH extremes that would occur by direct addition of acid or alkali. The stems and roots were ground in pH 7 buffer and pellets collected from centrifugation. The pellets were washed with, and then resuspended in, 0.3 M sorbitol with no buffer. Aliquots of a resuspended pellet solution were added to 150 mM glutathione. Volumes were chosed such that the resulting solution, ready for assay, consisted of 100 mM buffer, 0.3 M sorbitol, and 1–1.5 mM glutathione at the desired pH.

*Details of Specific Experiments.* Conditions for preparing the crude extracts are listed in Table I. Details of centrifugation and preparing the enzyme extracts for assay are given in Table II.

## Results

*Development of Assay.* Critical to this study was the ability to detect accurately small conversions of reticuline to salutaridine. Methodology was developed to assay 0.1-nmol conversions with confidence. In a typical control experiment to determine assay limits, a 3-mL aliquot of plant homogenate, previously inactivated by heating in boiling water for 15 min, was treated with 10 nmol of [3-$^3$H]reticuline in buffer. After incubation for up to 2 h, acid and carrier reticuline and salutaridine were added. The alkaloids were recovered after pH

Table I: Conditions for Preparing Crude Extracts

| expt no. | material[a] | wt (g)/ assay | grinding method[b] | additives[c] |
|---|---|---|---|---|
| 1 | WP | 11 | MP | E |
| 2 | WP | 12 | MP | E, S |
| 3 | WP | 11 | MP | E, T2 |
| 4 | WP | 11 | MP | E, T2, S |
| 5 | SMR | 4 | MP | S |
| 6 | SMR | 22 | MP | S |
| 7 | SMR | 21 | MP | S |
| 8 | SMR | 12 | Bl | S, G |
| 9 | SMR | 8 | Bl | S, G |
| 10 | SMR | 15 | Bl | S, G, T5 |

[a] WP = whole plant; SMR = stems, midribs of leaves, and roots. [b] MP = mortar and pestle; Bl = blender; the grinding buffer was 100 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) adjusted with NaOH to pH 7.0. [c] E = EDTA, 5 mM; S = sorbitol, 0.3 M; T2 = thioglycolic acid, 2 mM; T5 = thioglycolic acid, 5 mM; G = glutathione, 1 mM.

Table II: Details of Fractionation of Crude Extracts

| expt no. | low-spin pellet formed at $g \times 10^{-3}$/ min | high-spin pellet formed at $g \times 10^{-3}$/ min | pellet wash soln (pH) | low-spin suspension media (pH) | high-spin suspension media (pH) |
|---|---|---|---|---|---|
| 5 | 1/10 | 10/10 | Mops[a] (7.0) | Mops (7.0) | Mops (7.0) |
| 6 | 1/10 | 10/10 | no wash | Mops (7.0) | Mops (7.0) |
| 7 | 1/10 | 10/10 | Mops (7.0) | | Mops (7.0) |
| 8, 9 | 2.5/15 | 20/15 | S[b] | | S |
| 10W | 2.5/15 | 30/15 | S | | Tris+[c] (9.0) |
| 10N | 6/15 | 30/15 | S | | Tris+ (9.0) |

[a] Mops at 100 mM. [b] S = 0.3 M sorbitol plus 1 mM glutathione. [c] Tris+ = 100 mM tris(hydroxymethyl)aminomethane hydrochloride plus 1 mM glutathione plus 5 mM thioglycolic acid.

adjustment and extraction with $CHCl_3$/2-propanol. Salutaridine was separated by TLC from the added reticuline and the codeine, morphine, and thebaine endogenous to the plant homogenate. By use of UV absorbance and scintillation counting, the total recovery and specific activity of salutaridine were determined. After normalization to account for losses during isolation and purification, salutaridine from control experiments contained typically only 0.1–0.3% of the activity originally added as [3-$^3$H]reticuline.

*Initial Studies on Whole Plant Extracts.* Preliminary experiments involving extracts of whole plants examined four parameters for effects on reticuline consumption and salutaridine production: (1) method of grinding the plants; (2) inclusion of a phenol oxidase inhibitor (thioglycolic acid) and, separately, (3) the effect of 0.3 M sorbitol in the extract buffer; (4) assay for enzyme activity in the presence of oxygen, hydrogen peroxide, and various cofactors.

All extracts were prepared in a glove box with a nitrogen atmosphere at 3 °C. These were made by homogenizing in a blender or by simple pulping with mortar and pestle. Additionally, other extracts were prepared by freezing the plant pieces in liquid nitrogen, grinding the frozen material to a powder, and allowing the plant material to thaw as buffer was added. The resulting particulate mixtures were filtered through Miracloth to give crude enzyme extracts.

Multiple assays, conducted to determine the best method of extract preparation, showed no consistent trend. Several experiments had radioactivity in the salutaridine fraction slightly higher than that of the controls. However, these results were independent of the grinding procedure since most of the extracts produced by each grinding method were inactive.

Table III: Results of Assays on Extracts of Whole Plants[a]

| expt no. | oxidant | additives[b] | % $^3$H not recovered as reticuline[c,d] | % $^3$H in salutaridine | % $^3$H in $H_2O$[c] (nonalkaloidal) |
|---|---|---|---|---|---|
| 1A | $O_2$ | none | 99.0 | 0.46 | 70.8 |
| 1B | $H_2O_2$ | none | 99.5 | 0.05 | 48.7 |
| 2A | $O_2$ | S | 99.6 | 0.13 | 72.4 |
| 2B | $H_2O_2$ | S | 99.7 | 0.32 | 69.8 |
| 3A | $O_2$ | T | 50.6 | 0.39 | 8.1 |
| 3B | $H_2O_2$ | T | 94.6 | 0.28 | 69.8 |
| 4A | $O_2$ | T, S | 47.2 | 0.43 | 3.1 |
| 4B | $H_2O_2$ | T, S | 45.6 | 0.54 | 6.5 |

[a] Plants were ground in 100 mM Mops buffer, pH 7.0, with mortar and pestle. [b] T = 2 mM thioglycolic acid; S = 0.3 M sorbitol. [c] Based on total activity of [3-$^3$H]reticuline added to enzyme extract. [d] Also referred to as percent reticuline consumed.

Assays of extracts prepared with mortar and pestle explored the effects of the three other variables. Previous experience indicated these poppy extracts would show high phenol oxidase activity. Attempts were made to reduce this activity and thereby avoid the production of quinones, which might inactivate the desired enzymes (Rhodes, 1977). The commonly applied phenol chelators, such as insoluble poly(vinylpyrrolidone) (PVP), were not used here to pretreat the extracts since the removal of endogenous phenolic substrates may have directed the remaining oxidases to more quickly degrade added [3-$^3$H]reticuline. Instead, inhibition of undesired phenol oxidase activity was attempted by using thioglycolic acid (TGA), which was initially chosen over other inhibitors since it was reportedly effective in lower concentrations (Rhodes, 1977). Oxygen and hydrogen peroxide were tested as oxidizing agents for the enzymic reaction.

Since reports have suggested alkaloid biosynthesis may be associated with membrane and organelle fractions (Fairbairn & Steele, 1981; Scott et al., 1978), we attempted to preserve organelles by using breaking buffers containing 0.3 M sorbitol. This was effective when single cells were isolated from *P. somniferum* (Paul & Bassham, 1977). The results from assays testing these variables are presented in Table III.

Low recoveries of intact [3-$^3$H]reticuline after the assays indicated substantial reticuline consumption, but often, much of the radioactivity was found in the nonalkaloidal aqueous fraction of the extraction. Reticuline degradation into unknown products occurred in assays using either oxygen (experiment 1A) or hydrogen peroxide (experiment 1B), and definite differences between the two conditions were revealed in assays with other additives. Reticuline consumption in the presence of oxygen occurred in extracts with or without sorbitol (experiments 1A and 2A) but was reduced by addition of TGA (experiment 1A compared to experiment 3A and experiment 2A compared to experiment 4A). In assays using hydrogen peroxide, loss of reticuline was not inhibited by TGA addition or by use of sorbitol alone (experiment 1B compared to experiment 3B and 2B). However, when both additives were applied to assays containing hydrogen peroxide, reticuline loss was markedly decreased (experiment 4B). In these assays, addition of hydrogen peroxide to the enzyme system was followed by gas evolution, suggesting the formation of oxygen from the hydrogen peroxide.

These observations were interpreted as the interaction of

Table IV: Enzymic Conversions by Fractions of Stem and Root Extracts

| expt no. | fraction | oxidant or $N_2$ | extract condition | % $^3H$ in salutaridine | % reticuline consumed |
|---|---|---|---|---|---|
| 5A | low spin[a] | $O_2$ | live | 0.6 | 44 |
| 5B | low spin | $N_2$ | live | 0.5 | 43 |
| 5C | low spin | $H_2O_2$ | live | 0.8 | 22 |
| 5D | high spin[b] | $O_2$ | live | 1.0 | 23 |
| 5E | high spin | $N_2$ | live | 2.3 | *d* |
| 5F | high spin | $N_2$ | HI[c] | 0.2 | 22 |
| 5G | high spin | $H_2O_2$ | live | 4.4 | 40 |
| 5H | high spin | $H_2O_2$ | HI | 0.3 | 26 |
| 5I | supernatant | $O_2$ | live | 0.4 | 44 |
| 5J | supernatant | $N_2$ | live | 0.4 | 37 |
| 5K | supernatant | $H_2O_2$ | live | 0.2 | 99 |

[a] Pellet collected after centrifugation at 1000*g* for 10 min. [b] Pellet collected after centrifugation at 10000*g* for 10 min. [c] HI = heat inactivated. [d] Instrumental problems prevented measurement.

two enzymic systems: a free, soluble phenol oxidase and a vesicle- or organelle-enclosed enzyme degrading hydrogen peroxide. Reticuline thus seemed to be degraded by phenol oxidases with oxygen. In some assays, oxygen was added directly while, in others, oxygen was derived from hydrogen peroxide by perhaps catalase released from organelles by osmotic shock (Tolbert, 1971).

In an attempt to enhance salutaridine accumulation to levels competitive with reticuline degradation, a mixture of materials likely to stimulate oxidative metabolism and to limit enzyme denaturation was applied. However, addition of the cofactors suggested in the literature (ATP, $MgCl_2$, NAD, CoA, and glutathione) (Fairbairn & Djote, 1970) led to no salutaridine production.

*Fractionation of Extracts from Stems and Roots.* Separation of the desired enzyme system from degradative activities was clearly necessary. Since phenol oxidase and catalase activities are particularly high in the chloroplast fraction (Mayer & Harel, 1979; Tolbert, 1970), the extracts were prepared from only stems, midribs, and roots. Centrifugation of latex (Fairbairn & Steele, 1981) and of tissue culture extracts (Scott et al., 1978) was reported to give pellets with alkaloid-related enzyme activity. Following these suggestions, we centrifuged the crude extract twice to give three fractions: (1) a low-spin pellet collected from 0*g* to 1000*g*, (2) a high-spin pellet collected from 1000*g* to 10000*g*, and (3) the supernatant. After the centrifugation, the pellets were resuspended in buffer and re-formed by centrifugation. Samples of the fractions were supplemented with the cofactors applied in the previous experiment and assayed in the presence of oxygen, hydrogen peroxide, or no oxidant at all.

The assays showed high levels of radioactivity in salutaridine (Table IV). Although the implied enzyme activity appeared in all centrifugal fractions, the greatest was found from the high-spin pellet and was enhanced with hydrogen peroxide. Here the radioactivity recovered as salutaridine was 4.4% that of the original [3-$^3$H]reticuline, nearly 18 times that of the heat-inactivated controls.

In the subsequent experiment (Table V), the pellets were not washed to expedite extract preparation and reduce possible time-dependent enzyme inactivation. Very little [$^3$H]salutaridine production was obtained.

When a new extract was prepared, the high-spin pellet was thoroughly washed with a large volume of fresh buffer before repelleting and performing assays. This apparently removed

Table V: Detection and Removal of Soluble Enzyme Inhibitors from High-Spin Pellet

| expt no. | fraction | pellet washed? | oxidant or $N_2$ | % $^3H$ in salutaridine | % reticuline consumed |
|---|---|---|---|---|---|
| 6A | low spin[a] | no | $H_2O_2$ | 0.3 | 42 |
| 6B | high spin[b] | no | $H_2O_2$ | 0.7 | 46 |
| 6C | high spin | no | $N_2$ | 0.2 | 17 |
| 6D | supernatant | | $H_2O_2$ | 0.2 | 97 |
| 7A | high spin[b] | yes | $N_2$ | 0.4 | 21 |
| 7B | high spin | yes | $H_2O_2$ | 4.1 | 37 |
| 7C | high spin (HI)[c] | yes | $H_2O_2$ | 0.1 | 20 |

[a] Pellet collected after centrifugation at 1000*g* for 10 min. [b] Pellet collected after centrifugation at 10000*g* for 10 min. [c] HI = heat inactivated.

Table VI: Effect of pH on Activity in High-Spin Fraction

| expt no. | pH[a] | buffer[b] | incubation time (min) | % $^3H$ in salutaridine | % reticuline consumed |
|---|---|---|---|---|---|
| 8A | 3.93 | NaOAc | 30 | 0.3 | 29 |
| 8B | 4.95 | NaOAc | 30 | 0.1 | 19 |
| 8C | 5.95 | Mes | 30 | 1.0 | 42 |
| 8D | 7.00 | Mops | 30 | 1.7 | 39 |
| 8E | 7.95 | Tris | 30 | 4.9 | 49 |
| 8F | 8.80 | Tris | 30 | 13.4 | 54 |
| 8G | 7.00 (HI)[c] | Mops | 30 | 0.3 | 21 |
| 9A | 6.89 | Mops | 30 | 0.4 | 35 |
| 9B | 7.38 | Mops | 30 | 0.4 | 33 |
| 9C | 7.97 | Tris | 30 | 1.1 | 39 |
| 9D | 8.34 | Tris | 30 | 0.9 | 38 |
| 9E | 8.99 | Tris | 30 | 0.9 | 39 |
| 9F | 9.36 | Tris | 30 | 1.1 | 35 |
| 9G | 9.86 | lysine | 30 | 0.9 | 52 |
| 9H | 10.75 | lysine | 30 | 0.8 | 47 |
| 9I | 8.99 (HI) | Tris | 30 | 0.3 | 23 |
| 9J | 9.86 (HI) | Tris | 30 | 0.5 | 56 |
| 9K | 8.34 | Tris | 60 | 2.2 | 34 |
| 9L | 8.99 | Tris | 60 | 3.8 | 38 |

[a] pH measured directly after conclusion of incubation. [b] Final concentrations in extract: buffer, 100 mM; sorbitol, 0.3 M; glutathione, 1 mM; Mes = 2-(*N*-morpholino)ethanesulfonic acid adjusted with NaOH. [c] HI = heat inactivated.

the critical source of enzyme inactivation since good conversion activity was restored. Unlike the initial studies (experiment 5), however, no activity was obtained without added oxidant, and high activity was obtained with added hydrogen peroxide.

An attempt was made to enhance activity by solubilization of the high-spin pellet in 1% Triton X-100 solution. Although this procedure was reported to increase alkaloid enzyme activity (Scott et al., 1978), here such a treatment destroyed all activity.

*Defining Conversion Conditions.* In order to enhance conversion of reticuline to salutaridine in the high-spin fraction, it was necessary to determine the effect of added cofactors and to specify the optimum pH and incubation period for the assays.

Omitting exogenous ATP, $MgCl_2$, NAD, and CoA from the assay solutions did not effect the level of [$^3$H]salutaridine accumulation. However, without glutathione or thioglycolic acid, the extracts discolored, implying increased phenol oxidase activity. Slightly less [$^3$H]salutaridine was recovered from these 15-min assays. Since subsequent experiments were to

Table VII: Assays of Extracts Prepared To Reduce Hydrogen Peroxide and Salutaridine Degradation Activities

| expt no. | incubation time (min) | % $^3$H in salutaridine | % reticuline consumed | % relative conversion[a] |
|---|---|---|---|---|
| 10W[b] | 0[d] | 0.2 | 5.2 | |
| | 8 | 19.0 | 20.9 | 92 |
| | 16 | 25.2 | 32.3 | 78 |
| | 32 | 45.3 | 60.0 | 75 |
| | 64 | 36.3 | 70.2 | 52 |
| 10N[c] | 0 | 0.2 | 2.0 | |
| | 8 | 11.2 | 13.2 | 85 |
| | 16 | 24.5 | 30.0 | 80 |
| | 32 | 40.4 | 47.4 | 85 |
| | 64 | 13.7 | 53.2 | 26 |

[a] Conversion of consumed [3-$^3$H]reticuline to [$^3$H]salutaridine. [b] Extract was prepared from the high-spin pellet collected from a wide centrifugation range (2500–30000*g*); Tris buffer, pH 9; protein content 4.05 mg/3-mL assay. [c] Extract from the high-spin pellet of a separate portion of the same extract; Tris buffer, pH 9. This pellet was collected over a narrower centrifugation range (6000–30000*g*); protein content 2.79 mg/3-mL assay. [d] Assay extract inactivated with 50 μL of 85% $H_3PO_4$ before [3-$^3$H]reticuline addition.

test much longer assay incubation periods, these thiol reagents were retained as assay additives.

The optimum pH for plant extraction and enzyme activity were investigated. Plants were ground in neutral or slightly acidic buffers to reduce possible problems from oxidation of deprotonated phenols. However, the resulting crude extracts showed very little reticuline to salutaridine conversion. High enzymic transformation occurred, however, as the pH of the extract was readjusted to 8–10 (Table VI). At pH ≥9.9, enzymic action was accompanied by [3-$^3$H]reticuline instability. Control experiments at these pHs with heat-inactivated extracts showed higher contamination of salutaridine with radioactive impurities. Normal background levels were obtained from control assays at pH ≤9. Extracts at pH 8.3 and 9.0 gave similar results in the 30-min incubations but showed a clear difference when the incubation was extended to 60 min. Later experiments were conducted under the most favorable condition, pH 9.

Attempts to determine the optimum duration of incubation revealed that the conditions for extract preparation were of great influence. Initial time studies were performed on a dilute extract obtained by gentle grinding of a small mass of plant material. The extract was portioned and assays made by using a sequence of times. [$^3$H]Salutaridine in these incubations steadily accumulated over a 1-h period but only to a low level.

In tests on a concentrated extract prepared by vigorous homogenization of a larger quantity of plant material, however, [$^3$H]salutaridine accumulated to its highest level within 8 min and then steadily declined. Hydrogen peroxide destruction was also apparent in this concentrated extract. Upon hydrogen peroxide introduction, immediate gas evolution began and lasted for about 5 min. Additional hydrogen peroxide was added after 30 min, and although more gas evolution was observed, the level of [$^3$H]salutaridine increased and the trend toward decreasing [$^3$H]salutaridine at earlier time points was reversed.

Another extract was prepared and divided into two portions. In the first portion, the pellet was collected between 2500*g* and 30000*g* (experiment 10W). In the other, the pellet was collected from 6000*g* to 30000*g* (experiment 10N). The protein content of the first pellet was 38% greater than that of the second, which was about 1 mg/mL. The enzyme extracts prepared by resuspending these pellets were used for another study of [$^3$H]salutaridine production as a function of incubation time.

Both enzyme extracts showed significant enzyme activity (Table VII). In the 30-min incubations, conversions of [3-$^3$H]reticuline to salutaridine reached levels of 40–45%. When corrected for reticuline recovered, 80–85% of the radioactivity of consumed [3-$^3$H]reticuline was recovered as salutaridine. The prolonged exposures in the 60-min incubations, however, resulted in some [$^3$H]salutaridine loss. In the extract from the narrower centrifugation range, overall reticuline consumption was less but salutaridine consumption from 30 to 60 min was greater.

*Characterization of Enzymatically Produced [$^3$H]Salutaridine.* A portion of the mixture of labeled and carrier salutaridine isolated after a 32-min incubation (Table VII) was analyzed further by reverse-phase HPLC. The UV absorption at 243 nm was recorded as fractions were collected for radioactivity analysis by liquid scintillation. All of the radioactivity injected was recovered in the eluted fractions. Those fractions associated with the salutaridine peak on the UV trace of the HPLC contained 96% of the injected radioactivity.

The rest of the salutaridine isolated by TLC (452 μg, 1.38 μmol) was converted to thebaine in 38% yield by reduction with sodium borohydride and treatment with thionyl chloride in pyridine (Sohar & Schoenewaldt, 1975). The synthetic thebaine, purified by silica TLC, had $R_f$ and UV absorption values identical with those of natural thebaine. Within experimental error, the specific activities of the starting salutaridine and the resulting thebaine were the same (1123 ± 82 dpm/μmol compared to 1043 ± 27 dpm/μmol).

## Discussion

The enzyme preparation described here provides the first example of high-yield, plant-free conversion of reticuline to salutaridine. Success is attributable to three considerations. First, studies began with extracts from whole plants since only in plants had previous investigators reported high and specific enzyme activity, as indicated by tracer feeding studies.

Second, the assay monitored directly the conversion of radiolabeled substrate to product. This avoided the ambiguity that could arise from the measurement of secondary responses, such as oxygen consumption or more easily analyzed products further along the metabolic pathway. The high sensitivity and reproducibility of this assay allowed significant interpretations of results showing only slight variations.

Finally, it was critical that the assay design also permitted the assessment of competing degradative activities. Tracing radioactivity from the substrate into the nonalkaloidal aqueous fraction showed that the failure to detect product was not simply due to low activity of the desired enzyme system. Knowing whether either or both the substrate and the product were undergoing undesired transfromations allowed alternative steps to be taken.

In this manner, it was possible to balance efforts to preserve and to purify the enzyme activity. We had anticipated that the first major problem would be insufficient enzyme stability, as is often encountered in the initial stages of isolating plant proteins. Instead, the analyses of the fate of [$^3$H]reticuline indicated our first effort should be to reduce undesired enzyme activities competing for the substrate. It seemed likely that both reticuline and salutaridine could be attacked by phenol oxidases (Mayer & Harel, 1981). Attempts to limit oxidation involved addition of inhibitors in the grinding and incubation media (Rhodes, 1977) and reduction of the oxygen sources.

Exposing the extract to oxygen was lessened by preparing and assaying the extracts in nitrogen atmospheres. However, addition of hydrogen peroxide to the assay mixtures of the whole plants led to gas evolution, which was probably oxygen created by catalase action. Since high catalase activity is associated with the peroxisomes of photosynthetic tissues, extracts were later prepared from plant material without leaf tissue. This was useful but not expected to be totally effective since the stems and midribs of these plants contain some green tissue. Catalase is also associated with other organelles that occur in various plant tissues (Tolbert, 1971).

Centrifuging the stem and root extracts in addition was a more promising means of avoiding degradative activities. The supernatant was expected to contain phenol oxidase activity, since the phenol oxidases are often found as soluble intracellular components or as materials easily dissociable from membranes (Mayer & Harel, 1979). In our studies, the supernatant fractions showed extensive [3-$^3$H]reticuline consumption without appreciable salutaridine production.

In the low-spin pellets were sedimented the bulk of the pigmented particles, such as cell wall debris, chloroplasts, chloroplast and peroxisome aggregates, and intact peroxisomes (Price, 1970; Tolbert, 1970). In assays of the resuspended low-spin pellet, little [$^3$H]salutaridine accumulated. The desired enzyme system may have been present in only small quantities, or degradative action on both hydrogen peroxide and the alkaloids may have occurred by catalase and oxidases released from rupturing peroxisomes and chloroplasts during the relatively long incubation periods.

Enzymatic conversion of reticuline to salutaridine was extensive in the high-spin fraction, although small amounts of chloroplastic material and occasionally catalase-type activity were also observed here. Washing the high-spin pellet was necessary to obtain high levels of reticuline to salutaridine conversion. This may have removed an enzyme inhibitor, but it is as likely that the washing simply reduced competing degradative activities from adsorbed catalase and phenol oxidases. Adsorption of these enzymes after plant homogenization onto insoluble materials has long been recognized as a major difficulty in plant organelle isolation and characterization (Zelitch, 1964; Tolbert, 1971). The combination of not only washing the pellet but also using initially a gentle plant grinding technique to prevent rupture of organelles and enzyme dispursion and adsorption was the approach applied to the later experiments.

In studies to enhance enzyme activity by adjustment of the pH, it was surprising to find an optimum at 9 and reasonable activity at even higher pHs. However, enzymes may be particle enclosed and function at a pH quite different from that of the external buffer. At pH 8–9, reticuline is at its most lipophilic state, as demonstrated by its extractability from aqueous solutions into organic solvents. The observed pH enhancement may simply reflect concentration of the substrate by solubilization into lipophilic materials of the high-spin pellet.

Of primary importance now is the study of the stereochemical specificity of this enzyme reaction. The oxidative coupling of reticuline to salutaridine establishes the stereochemistry at the quaternary carbon (C-13) and hence the stereochemistry of the morphinan alkaloid skeleton. Since the morphinan alkaloids obtained from the opium poppy are a single enantiomer, one would expect reticuline to salutaridine conversion in these extracts to be stereospecific.

In our experiments, (±)-[3-$^3$H]reticuline was added to the enzyme extract, and in the experiments of highest conversion, approximately half of the [3-$^3$H]reticuline was consumed. This suggests the conversion of only one reticuline isomer. Tracer studies in plants, however, indicated that both isomers of reticuline are incorporated into (−)-morphine. The interconversion of (+)- and (−)-reticuline is accomplished through the intermediacy of the 1,2-dehydroreticulinium ion (Battersby et al., 1965; Borkowski et al., 1978). The possible interaction within a cell-free extract of a reticuline-racemizing system with a stereospecific system that converts (−)-reticuline to (+)-salutaridine is now under study.

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