

## myo-Inositol Oxygenase from Hog Kidney

### I. PURIFICATION AND CHARACTERIZATION OF THE OXYGENASE AND OF AN ENZYME COMPLEX CONTAINING THE OXYGENASE AND D-GLUCURONATE REDUCTASE\*

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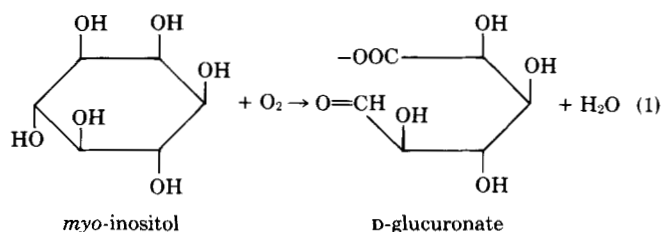
C. Channa Reddy‡, James S. Swan, and Gordon A. Hamilton§

From the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Homogeneous *myo*-inositol oxygenase (EC 1.13.99.1) has been obtained in 45% yield by a 550-fold purification from a centrifuged homogenate of hog kidney. Of particular importance in developing the purification procedure was the finding that although the enzyme becomes less catalytically active during purification, it could be reactivated by incubating with 1 mM Fe(II) and 2 mM cysteine. The molecular weight of the homogeneous oxygenase, as determined by 3 independent methods, is  $65,000 \pm 1,000$ , and no evidence could be found for the existence of any smaller subunits. The specific activities of homogeneous preparations vary, and this was found to correlate with the iron content; the most active preparations have 4 atoms of iron/65,000 daltons.

The homogeneous oxygenase has kinetic characteristics (pH maximum at 6.0) different from those (pH maximum at 8.0) of the oxygenase present in crude extracts. By modifying the purification procedure, another form of the oxygenase, which retains the kinetic characteristics of the enzyme in the homogenate, was purified 300-fold in 36% yield. Analytical gel electrophoresis indicates that 1 migrating species accounts for about 90% of the protein in the purified preparation. This labile species, which has a molecular weight of approximately 250,000, was shown to be an enzyme complex, which contains not only the oxygenase, but also D-glucuronate reductase (EC 1.1.1.19) and at least 2 other unidentified proteins. In its native state, the oxygenase is believed to exist as part of this complex.

In animals, the first committed step in the metabolism of *myo*-inositol occurs exclusively in the kidney and involves the cleavage of the ring to give D-glucuronic acid (1-3) as shown in Equation 1. In subsequent steps, the D-glucuronate is successively converted into L-gulonate, 3-keto-L-gulonate, L-xylulose, xylitol, D-xylulose, and D-xylulose 5-phosphate which then enters the pentose phosphate cycle (4, 5). *myo*-Inositol oxygenase (EC 1.13.99.1), which catalyzes the initial reaction (Equation 1) of this pathway, has been previously purified from rat kidney (2, 6) and oat seedlings (7), but, because the enzyme isolated from these sources is very unstable, not much



information is available concerning its detailed characteristics and concerning the mechanism of the reaction.

Although purified oxygenase preparations have received relatively little study, the reaction is of considerable interest physiologically. Weinhold and Anderson (8) observed that inhibition of the enzyme *in vivo* leads to rapid and early death from kidney failure, but for reasons which are not clear. For some time it has been known that individuals with diabetes mellitus excrete excessive amounts of inositol in their urine (9), and recent evidence indicates this is due to a decreased activity of *myo*-inositol oxygenase (10). The alterations in tissue levels of inositol in diabetics are believed to contribute to diabetic neuropathy (10) and to infantile respiratory distress (11).

As part of our continuing interest in the mechanisms of biological oxidation-reduction reactions (12, 13), we have recently begun a detailed investigation of the mechanism of the inositol oxygenase reaction. Because of the demonstrated instability of the rat kidney enzyme (2, 6), we initially surveyed homogenates of other animal kidneys and found that the enzyme in hog kidney appeared to be more easily stabilized. Consequently, we have developed a method for the purification of the hog kidney enzyme to homogeneity and have determined several of its characteristics. One notable feature of the homogeneous enzyme is that it is maximally active at pH 6.0, while the enzyme present in crude homogenates has a pH maximum at approximately 8.0. By modifying the purification procedure, it was possible to obtain a nearly pure preparation of a complex of several proteins, one of which is the oxygenase and another of which is NADPH-linked D-glucuronate reductase (EC 1.1.1.19). This enzyme catalyzes the next step in the pathway of inositol catabolism, namely, the conversion of D-glucuronate to L-gulonate. The oxygenase in this complex has kinetic characteristics (pH maximum at 8.0) similar to that in crude homogenates so presumably this form is the native state of the enzyme.

In the present article, we report the methods developed for obtaining both the homogeneous oxygenase and the complex and describe many of their characteristics, especially those of the homogeneous enzyme. In addition, since D-glucuronate reductase co-purifies with the oxygenase through several steps, a simple method for obtaining this enzyme in a near

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‡ Present address, 226 Fenske Laboratory, The Center for Air Environment Studies, The Pennsylvania State University, University Park, PA 16802.

§ To whom correspondence should be addressed.

homogeneous state is also described. In an accompanying article (14), some kinetic characteristics of the homogeneous oxygenase and the effects of various additives on its catalytic rate are reported.

#### EXPERIMENTAL PROCEDURES<sup>1</sup>

##### RESULTS

**Purification Procedures**—In early attempts to develop a purification procedure for the hog kidney oxygenase, assay conditions similar to those used by Charalampous (1, 2) were employed. Although good activity was observed in crude homogenates, it was frequently observed that nearly all of the catalytic activity, measured using these assay conditions, is lost when the enzyme is subjected to relatively mild purification conditions, especially ion exchange chromatography at pH 6 to 7. In subsequent experiments, it was found that this catalytically inactive enzyme could be reactivated by incubating with 1 mM Fe(II) and 2 mM cysteine. Thus, by using these components in the assay medium, it became possible to locate the enzyme in various fractions following purification steps, and a method was developed to readily purify it approximately 550-fold in 45% yield from the supernatant ( $105,000 \times g$ ) of a crude homogenate (Table I). Especially effective steps in the purification procedure are chromatography on a DEAE-cellulose (DE52) column and gel filtration on a Bio-Gel P-150 column, both performed at pH 6.0. The final chromatographic step using quaternary aminoethyl (QAE)-Sephadex at pH 6.0 removes some minor protein contaminants. The use of pH 6.0 acetate buffers for these steps is important; when similar steps were attempted using various buffers at pH 7.0, poor separations resulted, and large losses in catalytic activity were observed, especially with phosphate buffer. On the other hand, the preparation of crude extracts in pH 6.0 acetate buffers resulted in relatively low recoveries of catalytic activity. For these reasons, the final purification procedure involves using a pH 7.0 phosphate buffer for homogenization and ammonium sulfate precipitation and pH 6.0 acetate buffers for the remaining steps.

The results given in Table I show that whereas the oxygenase in the crude extract, and after ammonium sulfate fractionation, is nearly twice as active at pH 8.0 as it is at pH 6.0, the enzyme after subsequent purification steps is approximately 7 times more active at pH 6.0 than at pH 8.0. Such results imply that the enzyme is present in a different state when it is in crude extracts than when it is highly purified. Preliminary evidence that it may be present in the crude state as an aggregate of several proteins was obtained when it was observed that following ammonium sulfate fractionation, the enzyme in a buffer at pH 7.0 elutes from a Sephadex G-150 column near the void volume. By using pH 8.0 buffers rather than pH 6.0 ones for purification steps subsequent to the ammonium sulfate fractionation, it was possible to purify this protein complex 300-fold in 36% yield from a centrifuged crude extract (Table III). Negative adsorption on calcium phosphate gel and chromatography on a DEAE-Sephadex column are particularly effective steps in the purification. Throughout this purification procedure, the inositol oxygenase activity at pH 8.0 is always higher than that at pH 6.0; after the final

Bio-Gel P-200 gel filtration, the catalytic activity at pH 8.0 is 3 to 4 times that at pH 6.0 (Fig. 5). For convenience, the enzyme purified as summarized in Table I, and more active at pH 6.0 than at pH 8.0, will hereafter be referred to as the "pH 6 oxygenase," while that purified as summarized in Table III, and more active at pH 8.0 than at pH 6.0, will be referred to as the "pH 8 oxygenase complex."

It is evident from Figs. 4 and 5 that a considerable amount of NADPH-linked D-glucuronate reductase activity co-purifies with the pH 8 oxygenase complex. In the final preparation, the D-glucuronate reductase activity is 107 mkat<sup>2</sup>/kg compared to 24 mkat/kg for the oxygenase activity at pH 8.0. Since the reductase is considerably more active than the oxygenase, the oxygenase activity of the complex can be determined by following the change in absorbance at 340 nm when NADPH (0.25 mM) is present. The rate observed with inositol as substrate is identical with that obtained using the orcinol assay when the reaction is carried out under the same conditions but with NADPH absent. This is further evidence that the reductase and oxygenase are closely associated in the complex.

In the purification of the pH 6 oxygenase, all of the D-glucuronate reductase activity separates cleanly from the oxygenase during anion exchange chromatography on DEAE-cellulose at pH 6.0 (Fig. 1). One further step (gel filtration on Bio-Gel P-150) of purification of the reductase leads to a preparation which was estimated by analytical gel electrophoresis to be approximately 90% pure. This relatively simple purification procedure (summarized in Table II) is thus a convenient method for obtaining nearly homogeneous D-glucuronate reductase in good yield (43%).

**Stability of the Oxygenase Preparations**—The presence of reduced glutathione (1 mM) in the buffers is essential throughout purification and storage in order to avoid irreversible losses of oxygenase activity. The glutathione could not be replaced by other reducing agents, and most other thiols were also ineffective (cysteine was able to stabilize the enzyme to some extent). The presence of 50 mM KCl was also found to improve the stability of the oxygenase both during purification and storage. The pH 6 oxygenase is inactivated by ammonium sulfate precipitation so ultrafiltration was always used for concentrating this protein in the later stages of the purification.

If stored in a pH 6.0 acetate buffer (50 mM) containing 1 mM glutathione and 50 mM KCl, the pH 6 oxygenase is stable for months at  $-20^{\circ}\text{C}$ . By "stable," it is meant that the catalytic activity measured in the presence of 1 mM Fe(II) and 2 mM cysteine is constant. Although the pH 6 oxygenase can be frozen and thawed once with no appreciable loss of catalytic activity, repeated freezing and thawing leads to activity loss, i.e. as measured in the presence of Fe(II) and cysteine. At  $4^{\circ}\text{C}$ , the enzyme gradually loses activity over a few days and more rapidly if it has been frozen and thawed.

A systematic series of experiments to find optimum conditions for storing the pH 8 oxygenase complex has not yet been carried out, but some data have been gathered concerning this point. The partially purified preparation following calcium phosphate gel treatment can be stored at least 3 months at  $-20^{\circ}\text{C}$  in pH 8.0 buffer with little loss of pH 8 catalytic activity. However, the highly purified oxygenase complex gradually loses pH 8.0 activity over a period of days at  $-20^{\circ}\text{C}$  or  $4^{\circ}\text{C}$ , and sometimes a similar loss occurred during the final purification steps at  $4^{\circ}\text{C}$ . The presence of 50 mM KCl and 1 mM glutathione seemed to protect the pH 8.0 activity to some extent, but increasing the enzyme concentration was ineffec-

<sup>1</sup> Portions of this paper (including "Experimental Procedures," Figs. 1–13, and Tables I–V) are presented in miniprint at the end of the paper. Miniprint is easy to read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20814. Request Document No. 81M-341, cite authors, and include a check or money order for \$10.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

<sup>2</sup> The abbreviations used are: mkat, millikatal; SDS, sodium dodecyl sulfate.

tive. No conditions have yet been found for reconstituting the pH 8.0 activity after it is lost. Specifically, the addition of sulfhydryl compounds and/or ferrous ions has no effect in this regard. In all cases where it was checked, the loss of oxygenase activity at pH 8.0 leads to the generation of oxygenase activity at pH 6.0.

**Effect of Fe(II) and Cysteine on the Catalytic Activity of the Oxygenase Preparations**—As stated earlier, and as illustrated by the data in Table IV, the pH 6 oxygenase has very little catalytic activity measurable by the orcinol assay unless Fe(II) and cysteine are present. However, Fe(II) and cysteine are not necessary to obtain maximum activity with the pH 8 oxygenase complex when assayed at pH 8.0. In fact, the results suggest that Fe(II) and cysteine may increase the rate of decomposition of the complex to the pH 6 oxygenase. A more extensive investigation of the effects of various additives (including Fe(II) and cysteine) on the pH 6 oxygenase is given in the accompanying paper (14). A similar extensive series of experiments on the pH 8 oxygenase complex has not yet been carried out.

**Purity and Composition of the Oxygenase Preparations**—The freshly prepared pH 6 oxygenase (from Step V) elutes as a single symmetrical peak when reappplied to a Bio-Gel P-150 column, and the specific activity is the same in all of the protein-containing fractions. As illustrated in Fig. 6, only one protein band is seen on sodium dodecyl sulfate (SDS) gel electrophoresis and on analytical gel electrophoresis at pH 7.5. Furthermore, the enzyme activity is located at a position corresponding to the protein band on the analytical gels. Similar results are obtained when the electrophoresis is performed at pH 4.3 and at pH 8.3 and when the gels are overloaded with large amounts (25 to 50  $\mu$ g) of protein. These results indicate, therefore, that the enzyme is essentially homogeneous.

When a sample of the pH 6 oxygenase, which has been stored at  $-20^{\circ}\text{C}$  for 1 week or more, is subjected to analytical gel electrophoresis at pH 8.3, several protein bands are observed, one at the same position as seen with the freshly prepared enzyme, and some slower moving bands. SDS gel electrophoresis of a similar sample reveals only a single sharp band with the same mobility as obtained with freshly prepared enzyme. The results suggest that when the pH 6 oxygenase is stored at  $-20^{\circ}\text{C}$ , it polymerizes. However, the specific activities of the freshly prepared enzyme and of the stored enzyme are the same when assayed with Fe(II) and cysteine present.

One indication that the pH 8 oxygenase complex is near homogeneity is that the specific activities of both the oxygenase and the reductase are constant throughout the region that the complex elutes from the Bio-Gel P-200 column (Fig. 5). Further evidence for this conclusion was obtained on analytical disc gel electrophoresis. As shown in Fig. 7a, a freshly prepared sample of the pH 8 oxygenase complex gives only 1 major protein band, and this band contains both inositol oxygenase and D-glucuronate reductase activities. Approximately 90% of the protein on the gel is in this band so the preparation is about 90% pure.

Oxygenase activity at pH 8.0 is lost, and that at pH 6.0 appears when the pH 8 oxygenase complex is left at room temperature for several hours or is dialyzed overnight at  $4^{\circ}\text{C}$  versus a pH 6 buffer (Buffer C). When such a dialyzed sample of the pH 8 oxygenase complex is subjected to analytical gel electrophoresis, the pattern shown in Fig. 7b is obtained. At least 4 distinct protein bands are now evident. As indicated in Fig. 7b, 1 of the major protein bands has pH 6 oxygenase activity and a second major band has D-glucuronate reductase activity. These bands appear in the same regions as the pure proteins do when electrophoresed under the same conditions.

When the pH 8 oxygenase complex is subjected to SDS gel electrophoresis, at least 4 distinct protein bands (not illustrated) are also seen.

**Molecular Weights of the Oxygenase Preparations**—Three different methods for determining molecular weights all indicate that the pH 6 oxygenase has a molecular weight of  $65,000 \pm 1,000$ . As shown in Fig. 8, gel filtration of this enzyme along with marker proteins on a Sephadex G-150 column yields a molecular weight of 64,000. SDS gel electrophoresis of the enzyme and marker proteins in 10% polyacrylamide gels (Fig. 9) leads to a molecular weight estimate of 65,000. Inasmuch as this sample of the pH 6 oxygenase had been treated prior to electrophoresis with SDS and  $\beta$ -mercaptoethanol at  $100^{\circ}\text{C}$ , the enzyme is presumably not composed of smaller subunits held together by disulfide bonds. Data (Fig. 10) obtained from a sedimentation equilibrium experiment were used to calculate (15) a molecular weight of 66,000 for the pH 6 oxygenase.

Gel filtration of the pH 8 oxygenase complex and marker proteins on a Bio-Gel P-200 column leads to the results shown in Fig. 11. From these data, a molecular weight of approximately 250,000 for the intact complex, which was shown to have both D-glucuronate reductase activity and high inositol oxygenase activity at pH 8.0, is estimated. If the complex is broken up by dialysis versus a pH 6 buffer (Buffer C), then the oxygenase activity (measured at pH 6.0) elutes from a Bio-Gel P-200 column (not illustrated) at a position corresponding to a molecular weight of 65,000 and the reductase at a position expected for a protein of molecular weight approximately 32,000. These are well enough separated on such a column that the D-glucuronate reductase thus obtained was found to be homogeneous by analytical and SDS gel electrophoresis.

**Isoelectric Focusing of the pH 6 Oxygenase**—When the pH 6 oxygenase was subjected to isoelectric focusing, 2 components of equal intensity having pI values of 4.4 and 4.5 were observed, as shown in Fig. 12. These 2 charged isomers are not separated on gel electrophoresis under both denaturing and nondenaturing conditions. When the 2 components from the isoelectric focusing were eluted and tested for their inositol oxygenase activity, both had essentially the same specific activity.

**Specific Activity of the pH 6 Oxygenase**—During the course of the present research, the pH 6 oxygenase was purified several different times. Although the specific activity of any given preparation remained constant when stored at  $-20^{\circ}\text{C}$  for months, the specific activity of the various preparations ranged from 11 to 24 mkat/kg. In all cases, the purified preparations were judged to be homogeneous by analytical and SDS gel electrophoresis. Whether a preparation of high or low specific activity was obtained seemed to be determined by the activity in the crude homogenates rather than by some loss in activity during the purification procedure. Thus, approximately the same per cent yield and fold purification were obtained each time through the purification procedure; if the crude homogenate had a relatively low activity, then the homogeneous enzyme would have a low specific activity, and a homogeneous enzyme of high specific activity was obtained if the crude homogenate had a relatively high activity. The only difference in the various preparations which was detected is the amount of iron which each contains as described in the following section.

**Iron Content of the pH 6 Oxygenase**—As shown in Table V, the amount of iron present in different homogeneous preparations of the pH 6 oxygenase varies and appears to correlate with the specific activity of the preparation. The most active preparation has approximately 4 iron atoms/65,000 daltons, while less active preparations have as low as 1.5 atoms of

iron/molecule. No differences in the electrophoretic or chromatographic properties of the different preparations were detectable.

**Sulfhydryl Content of the pH 6 Oxygenase**—From the results shown in Fig. 13, one can calculate that 7 —SH groups/65,000 daltons of the intact enzyme are reactive toward *p*-chloromercuribenzoate, and 8 such groups react when the enzyme is present in 6 M urea. When similar titrations were performed using 5,5'-dithiobis(2-nitrobenzoate) as titrant (not illustrated), the numbers obtained were essentially the same.

**Optical Spectrum of the pH 6 Oxygenase**—The homogeneous enzyme has an absorption maximum at 278 nm and a minimum at 249 nm with extinction coefficients of 42,000 and 17,000 M<sup>-1</sup> cm<sup>-1</sup>, respectively (assuming a molecular weight of 65,000). In addition, considerable tailing in the absorption above 300 nm is observed but with no well defined peaks. At 350, 400, and 500 nm, the absorption is approximately 8%, 5%, and 2%, respectively, of that at 278 nm.

#### DISCUSSION

**The Homogeneous Inositol Oxygenase (pH 6 Oxygenase)**—Critical to the development of a procedure to purify the oxygenase to homogeneity was the finding that although the enzyme loses catalytic activity during the purification, it could be reactivated by incubating with 1 mM Fe(II) and 2 mM cysteine (14). The 5-step purification procedure developed as a result of this observation is a relatively convenient and simple one, and, thus, the enzyme is now readily obtainable. The purified oxygenase is homogeneous by the usual standard criteria.

Of considerable importance for subsequent work on the mechanism of the reaction is the finding that the hog kidney oxygenase can be stored indefinitely at -20 °C in a pH 6.0 acetate buffer with no appreciable loss in catalytic activity. This represents a major improvement over results obtained with the rat kidney enzyme which can be kept active for only a few hours or a couple of days at most (2, 6). A possible explanation for the notable instability of the purified rat kidney enzyme is that it may also exist in its native state as a protein complex, as found here for the hog kidney enzyme; it is reasonable that an unstable protein might result from the breakup of such a complex during purification.

The molecular weight of the hog kidney enzyme was found to be approximately 65,000 by several different methods. This is very similar to the reported molecular weights of the rat kidney (2, 6) and oat seedling (7) enzymes. Another common feature of the enzymes from the various sources is that they are all prone to polymerization either on storage (this research) or when the pH of the medium is altered (6, 7). One difference, however, between the rat and hog enzymes is that the hog enzyme appears to be a monomer, while the rat enzyme is a tetramer of 17,000 dalton units (6). No evidence for smaller subunits of the hog enzyme was obtained even when it was heated for extended periods of time with SDS and mercaptoethanol, conditions which lead to the smaller subunits being formed from the rat enzyme (6). Given the other close similarities between the rat and hog enzymes, and that the most active preparations of the hog enzyme contain 4 iron atoms/molecule (see below), it seems possible that the hog enzyme may be composed of 4 identical amino acid sequences of about 16,000 daltons, but which are covalently linked. Perhaps this also contributes to the greater stability of the hog enzyme when compared to that from the rat.

Another difference between the rat and hog oxygenases is their behavior on isoelectric focusing. Whereas the rat enzyme gives a single peak with an isoelectric point at pH 3.9 (6), the homogeneous hog enzyme separates into 2 catalytically active

components with isoelectric points at pH 4.4 and 4.5. Whether these charged isomers are an artifact of the purification procedure or are present as such in the physiological state remains to be determined.

Both groups of investigators who have worked with the rat kidney inositol oxygenase have reported that the enzyme contains iron, but the amount found per molecule of enzyme has varied. Charalampous (2) found 1 atom of iron/63,000 daltons, while Koller and Hoffmann-Ostenhof (6) reported that their preparations had more than 1 atom/65,000; they concluded that the catalytically active enzyme probably had 2 atoms. We observe (Table V) that the iron content of the hog enzyme varies from preparation to preparation, and it correlates with the specific activity of the homogeneous enzyme. The most active preparations have 4 atoms of iron/65,000 daltons. Since the iron content (and specific activity) of the enzyme seems to be fixed in the intact animal rather than being modified during enzyme purification, the lower activity of inositol oxygenase in diabetic animals (9, 10) could possibly be due to a decreased iron content of the enzyme.

The finding of 4 iron atoms/molecule in the most active preparations suggested that inorganic sulfide might be present in an iron-sulfur cluster. However, using the method of Gilboa-Garber (16), no inorganic sulfide could be detected (limit of detection, 0.3 atom/molecule) in the preparation shown to contain 4 iron atoms/molecule. Similar findings have been reported for the rat kidney enzyme (6). Although the hog kidney enzyme does not contain inorganic sulfide, it is a sulfhydryl enzyme, with 7 to 8 easily titratable —SH groups/molecule. Since the presence of 6 M urea has little effect on the number which is obtained, the titratable —SH groups are presumably on the surface of the molecule. Modification of these groups by sulfhydryl reagents leads to loss of catalytic activity (14).

No cofactor other than iron has been implicated in the inositol oxygenase reaction. Since the overall reaction catalyzed by the enzyme involves a glycol cleavage, and since chromium compounds are known to carry out such reactions (17), we considered the possibility that the enzyme might contain chromium, which is an essential trace element for animals (18). However, it was found by atomic absorption that there is no detectable chromium (limit of detection, 0.1 atom/65,000 daltons) in the homogeneous enzyme.

The optical spectrum of the hog kidney enzyme is similar to that reported for the rat enzyme (2) except that there is no well defined peak around 415 nm. Presumably, the diffuse absorption in the 300- to 400-nm region is due to an iron chromophore, but the lack of spectral detail precludes any conclusions at this time concerning the nature of the groups binding the iron.

**The Enzyme Complex (pH 8 Oxygenase Complex)**—The procedure reported here for obtaining in 90% purity an oxygenase-containing enzyme complex of molecular weight approximately 250,000 is relatively straightforward. The main difficulty in characterizing the complex is that the purified species is somewhat labile; it tends to decompose readily into its component proteins both on storage and during purification. Despite this problem, some initial characteristics of the complex have been determined.

Several pieces of evidence suggest that the complex represents the native state of the oxygenase. For example, the pH activity profile given by the oxygenase in the purified complex is similar to that (pH 8 maximum) observed using crude homogenates and quite different from that (pH 6 maximum) given by the homogeneous enzyme (14). Also, Fe(II) and cysteine are not required in order to observe catalytic activity in crude homogenates or with the purified complex, but are

required in order to observe high activity with the homogeneous enzyme. Although the purified complex is labile and breaks up into its component proteins readily, the oxygenase in the complex appears to be in a more stable state, *i.e.* does not require activation in order to show catalytic activity.

It is evident from the reported results that the purified enzyme complex contains not only inositol oxygenase but also NADPH-linked D-glucuronate reductase, the enzyme which catalyzes the second step in the pathway of inositol catabolism. In addition, the complex has at least 2 other proteins, but their identity and function are not yet known. It has been determined, however, that the complex does not contain NAD-linked L-gulonate dehydrogenase (EC 1.1.1.45), the enzyme which catalyzes the third step in inositol catabolism, namely, the conversion of L-gulonate to 3-keto-L-gulonate (19). Most of the L-gulonate dehydrogenase separates from the complex in the ammonium sulfate fractionation step and the rest during the calcium phosphate gel treatment.

Not all of the D-glucuronate reductase co-purifies with the oxygenase complex, but a major fraction of it does (Fig. 4). Since only 1 form of the reductase is observed after breakup of the complex during the purification of the pH 6 oxygenase, presumably the complex-bound reductase is the same enzyme as that which is not bound to the complex. Also, this enzyme is probably identical with the aldehyde reductase obtained from hog kidney by Bosron and Prairie (20). The specific activity of the purified enzyme toward D-glucuronate is comparable to that reported by Bosron and Prairie (20), and the molecular weight (approximately 32,000 as estimated by gel filtration) is similar to that previously found for the aldehyde reductase (20, 21). For some time there has been considerable controversy concerning the metabolic function of this relatively nonspecific aldehyde reductase. The finding in this research that a large amount of the activity is associated with the inositol oxygenase complex implies that, at least in kidneys, a major function of the enzyme is to catalyze the reduction of D-glucuronate.

A reasonable rationale for the close juxtaposition of inositol oxygenase and D-glucuronate reductase in biological systems can be offered, based on the suspected chemistry of the enzymic reactions and on the properties of D-glucuronate. In solution at equilibrium, D-glucuronate exists almost exclusively as a pyranose hemiacetal (22), but this would have to be converted to the acyclic aldehyde form before it could be transformed into L-gulonate by the reductase. The initial product of the oxygenase-catalyzed cleavage of myo-inositol is expected to be the free aldehyde, which should be a much better substrate for the reductase than equilibrated D-glucuronate ( $K_m$  approximately 8 mM) (20). Thus, with the oxygenase and reductase in the same complex, inositol could be converted to L-gulonate without the hemiacetal form of D-

glucuronate being an intermediate, and, therefore, D-glucuronate would not build up in solution.

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## SUPPLEMENTARY MATERIAL

TO

myo-Inositol Oxygenase from Hog Kidney. 1. Purification and Characterization of the Oxygenase, and of an Enzyme Complex Containing the Oxygenase and D-Glucuronate Reductase

BY

C. Channa Reddy, James S. Swan and Gordon A. Hamilton

## EXPERIMENTAL PROCEDURES

## Materials

Hog kidneys were obtained either from the Pennsylvania State University Meat Laboratory or from local slaughter houses. Orcinol, myo-inositol, NADPH, bovine serum albumin, reduced glutathione, cytochrome-C, hemoglobin, glucose-6-phosphate dehydrogenase, and alcohol dehydrogenase were obtained from Sigma Chemical Co. DEAE-cellulose (Whatman DE-52) was purchased from Reeve Angel, QAE-Sephadex (A-50) and DEAE-Sephadex (A-50) from Pharmacia, Biogel P-150 and ampholytes (Bio-lyte 3/10 and Bio-lyte 4/6) from Bio-Rad Laboratories, and electrophoretic materials (ultra pure grade) from Aldrich and Eastman. All the other chemicals used were reagent grade if not otherwise specified.

**Buffers Employed:** During protein purification and characterization several different buffers were used. In all cases 1 mM glutathione was present. Other components of the buffer solutions are: buffer A, 25 mM sodium phosphate, pH 7.0; 50 mM KCl; buffer B, 25 mM sodium acetate, pH 6.0; buffer C, 50 mM sodium acetate, pH 6.0; 50 mM KCl; buffer D, 25 mM sodium phosphate, pH 8.0; 50 mM KCl; buffer E, 25 mM Tris-HCl, pH 8.0; 50 mM KCl.

## Analytical Methods

**Protein Determination:** The protein concentration was determined by the method of Lowry et al (23) using bovine serum albumin as standard.

**Measurement of Oxygenase Activities:** Unless otherwise stated, a colorimetric procedure based on the reaction of orcinol with the product D-glucuronate (1,24) was employed. The standard assay mixture (2 ml) contained either 50 mM sodium acetate buffer pH 6.0, or 50 mM Tris-HCl buffer pH 8.0, 2.0 mM L-cysteine, 1.0 mM ferrous ammonium sulfate, 60 mM inositol, and appropriate quantities of enzyme (10 to 500  $\mu$ g). Prior to initiating the reaction by adding an aliquot of the substrate, the other reagents were incubated for 5 min at 30°C. After initiation, the reaction was allowed to proceed with shaking in an air atmosphere for 15 min at 30°C, and terminated by adding 0.2 ml of 30% trichloroacetic acid. Following removal of the precipitated protein by centrifugation, the amount of D-glucuronate formed was determined as previously described (1,24).

**Measurement of D-Glucuronate Reductase Activity:** The enzyme activity was determined spectrophotometrically at 30°C by following at 340 nm the rate of oxidation of NADPH during the reduction of D-glucuronate to L-gulonate. The reaction mixtures (1 ml) contained: 50 mM sodium phosphate buffer, pH 7.2, 0.2 mM NADPH, 10 mM D-glucuronate, and appropriate quantities of enzyme (5 to 50  $\mu$ g).

**Enzyme Activity Units:** All specific activities are reported in katal/kg protein; one katal is equal to one mole of product formed or substrate reacted per second.

**Polycrylamide Gel Electrophoresis:** Analytical disc gel electrophoresis was performed with 7.5% polycrylamide gels using tris-glycine buffer, pH 7.5, as described by Davis (25). A current of 2.5 mA per tube was applied until the bromophenol blue tracking dye front was 1 to 2 mm from the end. After running, the gels were either stained for protein detection or sliced into 1 mm slices for subsequent determination of enzyme activity. When stained, the gels were treated with Coomassie brilliant blue R-250 (0.1% in methanol: acetic acid: water; 5:7:87.5), destained electrophoretically in acetic acid: methanol: water; 7:10:83, and scanned at 610 nm using an ISCO recording monitor equipped with a gel scanning unit. The sliced gels were eluted with either buffer B (when the pH 6 oxygenase was subjected to electrophoresis) or buffer E (when the pH 8 oxygenase complex was so subjected), and aliquots of the eluate were used for enzyme assays.

**SDS Gel Electrophoresis:** Gels with 10% polyacrylamide (2.5% crosslinking) were prepared according to the method of Weber and Osborn (26). Prior to electrophoresis at 100 V per tube for 6 h, the proteins were incubated at 100°C for 10 min in a solution containing 1% SDS and 1%  $\beta$ -mercaptoethanol. The  $R_f$  values were calculated following staining and scanning as described in the previous section.

**Analytical Gel Filtration:** Gel filtration of the homogeneous pH 6 oxygenase was performed using a 1.5 x 110 cm column of Sephadex G-150 (40-120  $\mu$ m). A Bio-gel P-200 (50 to 100  $\mu$ m) column of the same size was used for gel filtration of the pH 8 oxygenase complex. Buffer B was used for column equilibration and elution of the Sephadex column, and buffer E for the Bio-gel column. In each case the enzyme plus marker proteins were applied with blue dextran to the column previously equilibrated with buffer, and 2.5 ml fractions were collected at a flow rate of 12 ml/h. The elution volume of reference compounds was determined by using literature procedures for their detection, and the molecular weight was estimated by a standard method (27).

**Analytical Ultracentrifugation:** An An-Ti rotor and a Spinco Model E analytical ultracentrifuge, equipped with a photoelectric scanner and ultraviolet optics, was used for sedimentation equilibrium analysis. Enzyme samples (0.4 mg/ml) dissolved in buffer B were centrifuged at 15,000 rpm and 27°C for 48 h. Double sector cells equipped with a charcoal filled Epon centerpiece and quartz windows were used to measure the absorbance at 280 nm. A partial specific volume of 0.73 ml/g was assumed in calculating the molecular weight.

**Isoelectric Focusing:** Polyacrylamide gels (5 x 125 mm) used for isoelectric focusing were prepared by photopolymerization (8.4  $\mu$ M FMN present) of 7.5% total monomer (with 2.5% of this as methylene bisacrylamide). Each gel also contained 5% (w/v) glycerol and 2% Bio-lyte (3/10 or 4/6). Enzyme samples (20  $\mu$ g) were applied in 25% sucrose solutions and overlaid with 10% sucrose. The gels were focused at 4°C for 20 h using a potential of 200 V. Cytochrome-c was present as a marker in one of the gels. Using the modification of Warden's procedure (28), the gels were stained in ethanol: water: acetic acid (50:45:5) containing 0.2% bromophenol blue, then destained and scanned at 560 nm as previously described. Blanks were run to determine the pH gradient along the gels at the end of the focusing.

**Determination of Iron Content:** In most cases the iron content of the enzyme was determined by atomic absorption using a Varian AA-5 spectrophotometer equipped with a Model 63 carbon rod atomizer and carbon furnace. In one case the colorimetric method of Landers and Zak (29) was employed using commercially available bathophenanthroline sulfonate (Sigma).

Special precautions were taken to avoid metal ion contamination of the enzyme samples. All H<sub>2</sub>O was doubly distilled, the second time using Kontes WS-2 glass still following percolation through Barnsted organic and ion exchange columns. All glassware was soaked overnight in concentrated HNO<sub>3</sub> and then rinsed 8 to 10 times with H<sub>2</sub>O. Pipetman with disposable plastic tips were used for all liquid transfers. The plastic tips were first soaked in 100 mM EDTA then rinsed thoroughly with H<sub>2</sub>O and dried in a vacuum desiccator. Injections (5  $\mu$ l) into the carbon tube furnace were performed using a 10  $\mu$ l syringe attached to a length of polyethylene tubing so that the solutions did not come into contact with the syringe needle. Fresh tubing was used for each solution, and it was rinsed 3 times with the solution to be injected before an injection was made.

An enzyme sample (0.5 ml) containing approximately 0.5 mg/ml was dialyzed 15 to 20 h versus 2 l of H<sub>2</sub>O at 4°C. After removing a small amount of precipitate by centrifugation, the supernatant was subjected to analysis for iron, catalytic activity, and protein content. The iron content of the dialysate was determined and used as a blank for the enzyme solution. Iron standards were prepared by diluting appropriate amounts of Fisher Scientific certified iron standard (1,000 ppm). Each reported enzyme determination was done on a different day using a different enzyme sample and freshly prepared iron standards. The iron content per mole of enzyme was calculated assuming a molecular weight of 65,000.

**Determination of Sulfhydryl Content:** In the following procedure all buffers used for dialysis of the enzyme sample were flushed with N<sub>2</sub> prior to use, and all containers were wrapped in aluminum foil or black cloth in order to exclude light. The enzyme sample (concentrated to 2.5 mg/ml by ultrafiltration) was dialyzed at 4°C for 12 h with 3 changes of a 50 mM sodium acetate buffer (pH 6.0) containing 2 mM glutathione. After centrifugation to remove insoluble material, the sample was redialyzed in a similar fashion using a 50 mM sodium acetate buffer (pH 6.0) which did not contain glutathione. Following centrifugation, the sulfhydryl content of the resulting solution was determined using p-chloromercuribenzoate as described by Boyer (30) and Benesch and Benesch (31), and using 5,5'-dithiobis (2-nitrobenzoate) as described by Ellman (32). The enzyme solution in 6 M urea was prepared by dialyzing the above solution versus buffer containing 6 M urea for 12 h at 4°C. In order to eliminate the high absorbance due to protein in the titrations, iodoacetamide treated enzyme was used in the reference cuvettes. To prepare this material, a sample of the dialyzed enzyme was incubated with 8 mM iodoacetamide at 24°C for 5 h, then dialyzed overnight at 4°C versus distilled water (3 changes), and centrifuged.

## Purification of Inositol Oxygenase to Homogeneity (pH 6.0 Activity)

**Step I: Preparation of Crude Extracts:** All purification steps were performed at 4°C. Hog kidneys, obtained within 10 min after the animals were killed, were immediately chilled in an ice bucket. The kidneys were minced (the connective tissue was cut away and discarded), washed with ice cold water, and the mince (500 g wet weight) homogenized in a Waring blender for 30 sec with 3 volumes (weight/volume) of buffer A. The extract was flushed with nitrogen gas and subjected to further homogenization in a Potter-Elvehjem teflon homogenizer. After centrifuging the resultant homogenate at 20,000 g for 30 min the pellet was discarded, and the supernatant was saved for further purification. At this stage of purification, addition of ferrous ammonium sulfate (to a final concentration of 1 mM) and fluxing with nitrogen promoted the stability of the enzyme during the course of purification. This enzyme solution was centrifuged at 105,000 g for 45 min, and the supernatant obtained was passed through glass wool in order to remove floating lipid materials. The reddish pink filtrate is referred to as the crude extract.

**Step II: Ammonium Sulfate Fractionation:** Solid ammonium sulfate was added to the enzyme solution from Step I to give a final concentration of 30%. After all the ammonium sulfate was dissolved, the pH of the solution was adjusted to 7.0 with dilute ammonium hydroxide, and the suspension was stirred for 1 h. The precipitated protein was removed by centrifugation at 20,000 g for 10 min and discarded. The resulting supernatant was brought to 45% saturation by further addition of solid ammonium sulfate, stirred for 1 hour, and then centrifuged at 20,000 g for 10 min. After carefully draining the supernatant off, the pellets were washed with saturated ammonium sulfate solution, and gently resuspended in buffer B to give a final volume of about 250 ml. This suspension was dialyzed overnight against 32 volumes of buffer B with one buffer change, and the dialyzed enzyme solution centrifuged at 20,000 g for 30 min in order to remove any insoluble materials present.

**Step III: DEAE-Cellulose (DE-52) Column Chromatography:** The enzyme fraction obtained from the previous step was applied to a DE-52 column (8 x 40 cm) previously degassed and equilibrated with buffer B. Using the same buffer as eluent, and a flow rate of approximately 90 ml/h, 10 ml fractions were collected until the absorbance at 280 nm had dropped to below 0.5 (a total of 0.9 to 1.0 l required). The eluting buffer was then changed to a linear KCl gradient prepared using 500 ml of buffer B and 500 ml of buffer B containing 0.25 M KCl, and a further 80 to 100 fractions were collected. As illustrated in Fig. 1, the D-glucuronate reductase activity elutes in the final stages of the elution with buffer B, and the inositol oxygenase comes off during the gradient elution at approximately 0.1 M KCl. For further purification of inositol oxygenase, the fractions containing oxygenase activity were pooled and concentrated to 15 ml by ultrafiltration through a UM-20 membrane in an Amicon concentration cell. No loss of enzyme activity occurred during concentration.

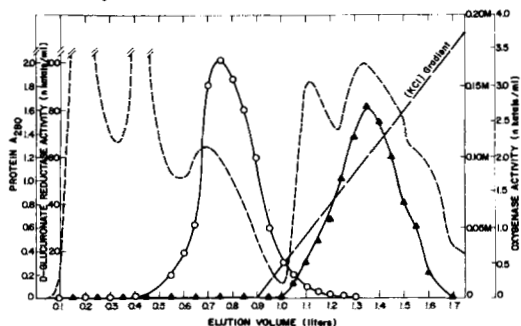


Figure 1. Chromatography of a dialyzed 30-45% ammonium sulfate fraction on a DEAE-cellulose (DE-52) column. Protein elution profile as indicated by the absorbance at 280 nm (—•—), D-glucuronate reductase activity (---○---), and inositol oxygenase activity at pH 6.0 (....Δ....).

**Step IV: Gel Filtration on a Bio-gel P-150 Column:** The oxygenase preparation (15 ml or less) from Step III was carefully loaded on to a Bio-gel P-150 column (4.2 x 110 cm) previously equilibrated with buffer C. Using the same buffer as eluent, 7.5 ml fractions were collected at a flow rate of approximately 60 ml per h. As shown in Fig. 2, the oxygenase elutes with a minor protein peak after most of the protein has come off. At this stage, analytical gel electrophoresis indicates that some of the fractions containing oxygenase activity are 90 to 95% pure.

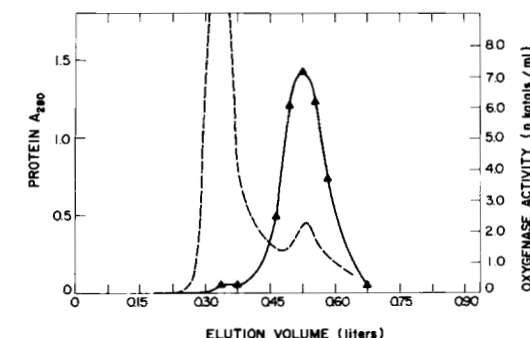


Figure 2. Gel filtration on a Bio-gel P-150 column of a concentrated inositol oxygenase preparation obtained from chromatography on a DEAE-cellulose column. Protein elution profile as indicated by the absorbance at 280 nm (—•—), and inositol oxygenase activity at pH 6.0 (---○---).

**Step V: QAE-Sephadex (A-50) Column Chromatography:** Active inositol oxygenase fractions from Step IV were applied to a precycled anion exchange Sephadex column (QAE-Sephadex A-50, 2 x 15 cm) which had been previously equilibrated with buffer C. When all the enzyme solution had entered the column, it was washed (flow rate, approximately 60 ml/h; 10 ml fractions collected) first with buffer C until no absorption of effluent at 280 nm could be detected (approximately 300 ml required). The absorbed enzyme was then eluted with a linear gradient of 400 ml of buffer C and 400 ml of buffer C containing 0.3 M KCl. As illustrated in Fig. 3, the oxygenase activity is well separated from the inactive impurities. The active fractions were pooled, concentrated by ultrafiltration (with several dilutions by buffer C to remove excess KCl) to a final concentration of approximately 0.5 mg/ml, and stored in 1 ml fractions at -20°C.

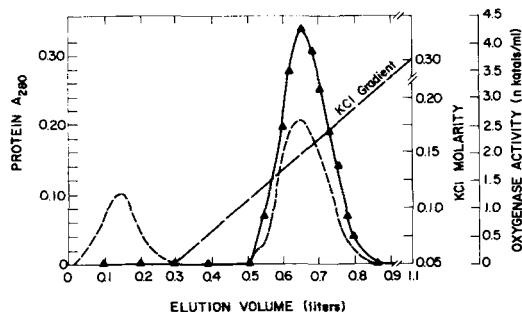


Figure 3. Anion exchange chromatography on a QAE-Sephadex column of the pooled oxygenase fractions from the Bio-gel column. Protein elution profile as indicated by the absorbance at 280 nm (—•—), and inositol oxygenase activity (pH 6.0) (Δ).

A summary of a typical purification procedure to give the inositol oxygenase which has maximum activity at pH 6.0 is given in Table I.

TABLE I

Summary of the purification of myo-inositol oxygenase (pH 6.0 activity) from hog kidney.

The enzyme was purified from 500 g (wet weight) of hog kidneys by the procedures described in the text.

Purification Step	Total protein (mg)	Total activity (μkatals) pH 6.0	Specific activity (mkat/kg) pH 6.0		Fold purification (pH 6.0 activity)	Yield (%) (pH 6.0 activity)
I. Crude extract	21,200	0.85	0.04	0.07	1	100
II. 30-45% ammonium sulfate fraction	8,200	0.66	0.08	0.14	2	77
III. DEAE-cellulose column	430	0.60	1.4	0.20	35	20
IV. Bio-gel P-150 column	32	0.44	13.8	2.0	340	50
V. QAE-Sephadex column	18.5	0.41	21.9	2.5	550	45

#### Purification of D-Glucuronate Reductase

As indicated by the results shown in Fig. 1, D-glucuronate reductase copurifies with inositol oxygenase through the first two steps but is separated when the DE-52 column (Step III) is run at pH 6.0 (buffer B). To obtain a nearly pure preparation of the reductase, fractions (Fig. 1) containing this activity were pooled, concentrated by ultrafiltration to 10 ml, dialyzed overnight versus 50 volumes of buffer D and subjected to gel filtration on a Bio-gel P-150 column previously equilibrated with buffer D. The protein fractions containing D-glucuronate reductase activity are well separated from higher molecular weight proteins which are inactive. The reductase thus obtained was found to be approximately 90% pure by analytical gel electrophoresis. A summary of a typical purification procedure to give the nearly homogeneous reductase is given in Table II.

TABLE II

Summary of the purification of D-glucuronate reductase from hog kidney.

The enzyme was purified from 500 g (wet weight) of hog kidneys by the procedures described in the text.

Purification step	Total protein (mg)	Total activity (μkat) pH 6.0	Specific activity (mkat/kg) pH 6.0	Fold purification (pH 6.0 activity)	Yield (%)
I. Crude extract	23,000	20.7	0.9	1	100
II. 30-45% ammonium sulfate fraction	8,400	14.6	1.7	2	70
III. DEAE-cellulose column	180	9.4	52	58	45
IV. Bio-gel P-150 column	48	8.9	185	206	43

#### Purification of the Inositol Oxygenase Complex (pH 8.0 Activity)

The preparation of the crude extract (Step I) and precipitation by ammonium sulfate (30 to 45% of saturation) were identical to procedures described for the preparation of the oxygenase with pH 6.0 activity. The precipitate was dissolved in 250 ml of buffer D, centrifuged for 24 h versus 25 volumes of buffer D (with 2 changes of buffer), and centrifuged at 15,000 g for 30 min. The clear brownish-yellow supernatant was designated as the ammonium sulfate fraction (Step II).

**Step III: Negative Adsorption on to Calcium Phosphate Gel:** After thoroughly washing and equilibrating calcium phosphate gel with buffer D, the fine slurry was slowly added (dry gel; protein, 1:1) with continuous stirring to the enzyme solution from Step II. The mixture was stirred for exactly 15 min and then the gel was removed by centrifugation at 6,000 g for 10 min. The gel was discarded and the supernatant treated a second time with calcium phosphate gel in a manner identical to that described above. The clear pale yellow supernatant thus obtained could be stored over a month at -20°C without appreciable loss of enzyme complex activity (oxygenase activity at pH 8.0).

**Step IV: DEAE-Sephadex (A-50) Column Chromatography:** After washing the DEAE-Sephadex (A-50) by the procedure of Peterson and Sober (33), and equilibrating with buffer E, it was packed in a column (4.2 x 46 cm), and again washed with several bed volumes of buffer E. The enzyme solution from Step III was applied to the column and the flow rate set at approximately 60 ml/h. After the protein sample had entered the column it was washed with buffer E until the effluent had an absorbance of less than 0.1 at 280 nm. Most of the protein applied to the column came off during this washing, but it had neither D-glucuronate reductase nor inositol oxygenase activity. The absorbed protein was eluted (10 ml fractions were collected) by a linear gradient of KCl (50 to 250 mM KCl with 750 ml of each component dissolved in buffer E). As indicated in Fig. 4, the oxygenase elutes at approximately 0.18 M KCl along with a considerable amount of the D-glucuronate reductase activity. The active oxygenase fractions were pooled and concentrated to 10 ml by ultrafiltration through a UM-20 membrane in an Amicon concentration cell. By analytical gel electrophoresis the enzyme preparation at this stage was estimated to be 60 to 70% pure.

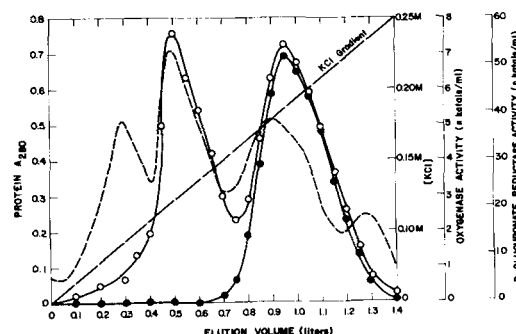


Figure 4. Chromatography on a DEAE-Sephadex (A-50) column of an enzyme preparation after negative calcium phosphate gel treatment. Protein elution profile as indicated by the absorbance at 280 nm (—•—), D-glucuronate reductase activity (○), and inositol oxygenase activity at pH 8.0 (●).

**Step V: Gel Filtration on a Bio-gel P-200 Column:** The enzyme preparation from Step IV was loaded on to a Bio-gel P-200 column (4.2 x 100 cm) previously equilibrated with buffer E, and eluted with buffer E at a flow rate of approximately 30 ml/h (10 ml fractions were collected). As illustrated in Fig. 5, most of the oxygenase active at pH 8.0 and most of the D-glucuronate reductase elute together immediately after the void volume. A small amount of reductase activity and oxygenase active at pH 6.0 elute along with other protein impurities in later fractions. The fractions containing both the reductase and oxygenase activities were pooled, concentrated by ultrafiltration (UM-20 filter) to approximately 5 mg/ml and stored in 0.5 ml fractions at -20°C.

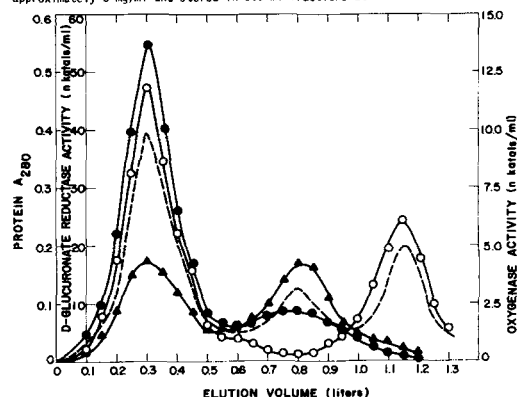


Figure 5. Gel filtration on a Bio-gel P-200 column of a concentrated oxygenase and reductase preparation obtained from chromatography on DEAE-Sephadex (A-50). Protein elution profile as indicated by the absorbance at 280 nm (—•—), D-glucuronate reductase activity (○), inositol oxygenase activity at pH 6.0 (Δ), and inositol oxygenase activity at pH 8.0 (●).

A summary of a typical purification procedure to give the enzyme complex containing D-glucuronate reductase activity and inositol oxygenase activity at pH 8.0 is given in Table III.

TABLE III

Summary of the purification of the myo-inositol oxygenase complex (pH 8.0 activity) from hog kidney.

The complex was purified from 500 g (wet weight) of hog kidneys by the procedures outlined in the text.

Purification step	Total protein (mg)	Total activity at pH 8.0 (μkat)	Specific activity at pH 8.0 (mkat/kg)	Fold purification (pH 8.0 activity)	Yield (%)
I. Crude extract	22,000	1.8	0.08	1	100
II. 30-45% ammonium sulfate fraction	8,100	1.3	0.16	2	72
III. Calcium phosphate gel	1,750	1.1	0.96	12	61
IV. DEAE-Sephadex column	58	0.8	13.8	173	44
V. Bio-gel P-200 column	27	0.65	24.1	301	36

TABLE IV

Effects of 1 mM Fe(II) and 2 mM L-cysteine on the activities of the inositol oxygenase preparations

The catalytic activities were determined using the orcinol assay at 30°C as described in the text.

Enzyme preparation	Specific activity (mkatals/kg)			
	without Fe(II) and Cys pH 6.0	without Fe(II) and Cys pH 8.0	with Fe(II) and Cys pH 6.0	with Fe(II) and Cys pH 8.0
pH 6 oxygenase	2.7	1.4	22.6	3.5
pH 8 oxygenase complex	3.1	24.8	6.2	21.6

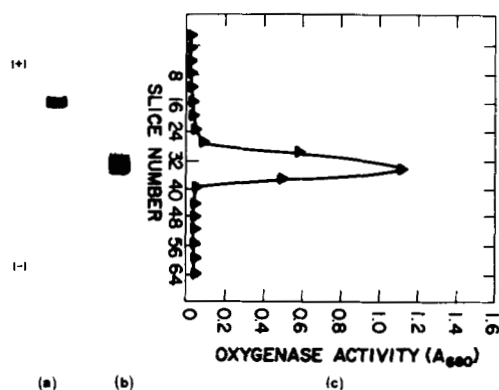


Figure 6. Electrophoresis of the pH 6 oxygenase. (a) SDS gel electrophoresis, (b) analytical gel electrophoresis at pH 7.5, and (c) profile of inositol oxygenase activity at pH 6.0 following analytical gel electrophoresis at pH 7.5. The enzyme activity is given by the absorbance at 660 nm generated in the standard orcinol assay by enzyme eluted from the various slices.

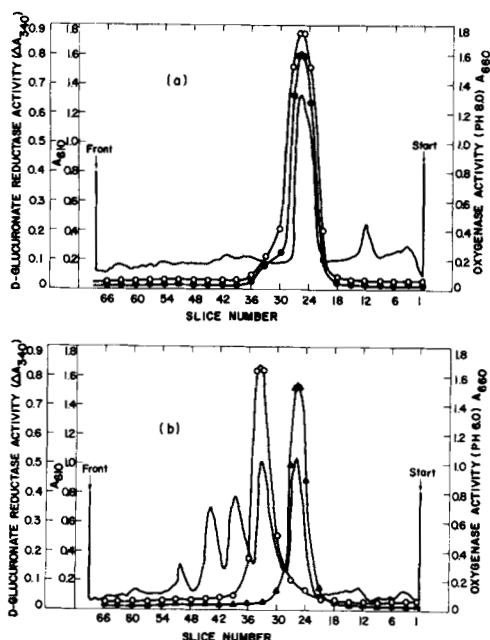


Figure 7. Analytical gel electrophoresis at pH 8.3 of (a) freshly prepared pH 8 oxygenase complex, and (b) pH 8 oxygenase complex which had been dialyzed overnight at 4°C versus buffer C. The amount of stained protein (—) was determined by gel scanning and is given by the absorbance at 510 nm, the D-glucuronate reductase activity (○) is given as the change in absorbance per min at 340 nm using enzyme eluted from the various slices, and the inositol oxygenase activity at pH 8.0 (●) or 6.0 (▲) is given by the absorbance at 660 nm generated in the standard orcinol assay by enzyme eluted from the various slices.

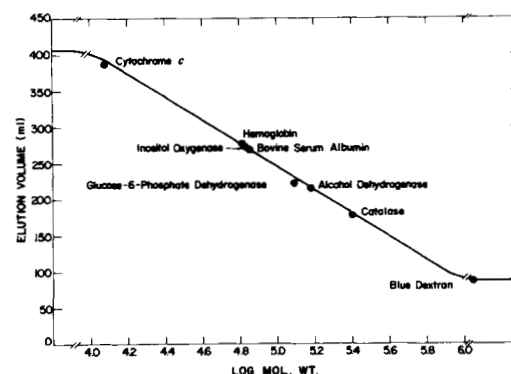


Figure 8. Molecular weight determination of the pH 6 oxygenase by gel filtration on a Sephadex G-150 column. For further details see the experimental section.

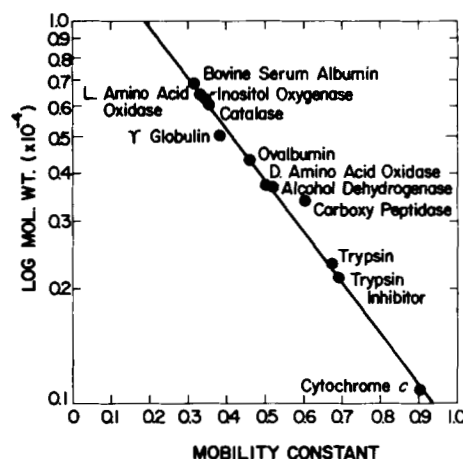


Figure 9. Estimation of the molecular weight of the pH 6 oxygenase by gel electrophoresis using 10% polyacrylamide gels and 0.1% SDS. Detailed conditions are given in the experimental section.

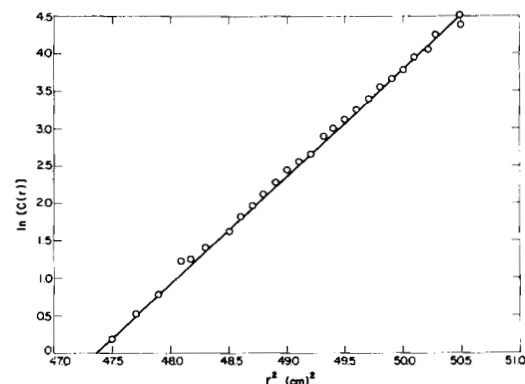


Figure 10. Sedimentation equilibrium data obtained using the pH 6 oxygenase in buffer C. The distance from the axis of rotation is given by  $r$ , and  $c(r)$  is the protein concentration at that distance. For other details see the experimental section.



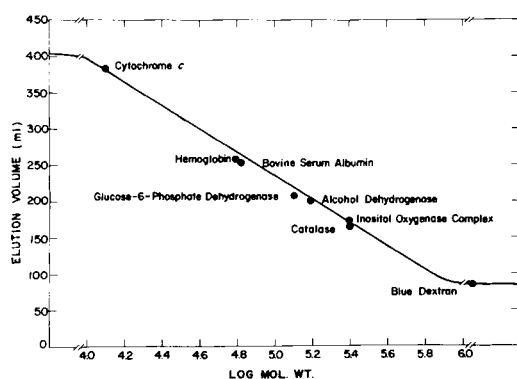


Figure 11. Molecular weight determination of the pH 6 oxygenase complex by gel filtration on a Bio-gel P-200 column. For further details see the experimental section.

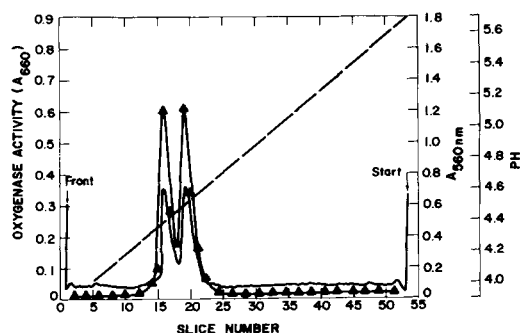


Figure 12. Isoelectric focusing of the pH 6 oxygenase. The amount of stained protein (—) was determined by gel scanning and is given by the absorbance at 560 nm, and the inositol oxygenase activity at pH 6.0 (•) is given by the absorbance at 660 nm generated in the standard orcinol assay by enzyme eluted from the various slices. The pH is given by the dashed line (----).

TABLE V

The iron content of the pH 6 oxygenase and its correlation with specific activity

The iron content was determined by atomic absorption spectroscopy except where noted. Each reported value was obtained with a different sample of enzyme; the reported variation in each value is the range observed in several determinations with the same enzyme sample. For other details see the experimental section.

Enzyme preparation	Specific activity at pH 6.0 ( $\mu$ kat/kg)	Moles iron per mole enzyme
I	23	$4.0 \pm 0.2$ $3.7 \pm 0.3$
II	11	$1.5 \pm 0.1$ $1.4 \pm 0.2^a$
III	12	$1.5 \pm 0.1$ $1.8 \pm 0.1$

<sup>a</sup>Determined colorimetrically by the method of Landers and Zak (29).

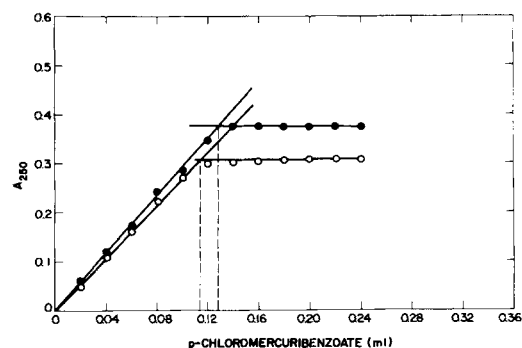


Figure 13. Titration of the pH 6 oxygenase with p-chloromercuribenzoate. The absorbance changes were measured 10 min after each addition at 24°C of an aliquot of a 0.46 mM stock solution of p-chloromercuribenzoate to 3.0 ml of a solution of the pH 6 oxygenase (2.5  $\mu$ M) in 50 mM sodium acetate buffer, pH 6.0 (O), or in the same buffer containing 6 M urea (●).