$K^+$. In any case, with our results it is not possible to rule out a small participation of $K^+$.

D. J. CHIARANDINI*
E. STEFANI†
H. M. GERSCHENFELD‡
*Instituto de Anatomía General y Embryología, Facultad de Ciencias Médicas, Buenos Aires, Argentina*

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* Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. Present address: Laboratory of Neurophysiology, Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, N.Y.
† Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. Present address: Department of Biophysics, University College, London, England.
‡ Present address: Department of Neurobiology, Harvard Medical School, Boston, Mass.

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## Sulfite Oxidase Deficiency in Man: Demonstration of the Enzymatic Defect

Abstract. *An infant who died with neurological abnormalities, mental retardation, and dislocated ocular lenses excreted in his urine abnormally large amounts of* S-*sulfo*-L-*cysteine, sulfite, and thiosulfate and virtually no inorganic sulfate. The present report establishes the occurrence of an enzymatic defect in this infant. His liver, brain, and kidney specifically lacked sulfite oxidase activity. Deficiency of sulfite oxidase, which has not apparently been described in man, provides a reasonable explanation for the abnormalities in this infant.*

We have described (*1*) a male infant with a disorder of metabolism which we believe was previously unrecognized. At birth he had neurological abnormalities which progressed until he was virtually decerebrate. Dislocated ocular lenses were noted at age 1 year. He died at 32 months. Three of seven siblings were born with neurological abnormalities and died in early infancy for undefined reasons. The remaining siblings and the parents appear normal on clinical examination and have not yet been shown to have any disorders of their metabolism. The patient's urine contained greatly elevated concentrations of *S*-sulfo-L-cysteine, sulfite, and thiosulfate and virtually no inorganic sulfate (*2*). The abnormal chemical findings are readily reconciled by postulation of a deficiency of activity of sulfite oxidase, the enzyme that catalyzes the conversion of sulfite to inorganic sulfate. In the presence of such a deficiency, sulfate excretion would be abnormally low, and sulfite would accumulate. The raised concentration of sulfite in body fluids could lead secondarily, by way of known reactions, to the formation of abnormal amounts of *S*-sulfo-L-cysteine (*3*) and thiosulfate (*4*). We now report direct evidence that the patient was indeed markedly deficient in sulfite oxidase activity.

Sulfite oxidase [sulfite : oxygen oxidoreductase E.C. 1.8.3.1 (*5*)] of mammals has been studied by Fridovich and Handler and their associates (*6*, *7*). Their methods, with minor modifications, were used for our study of tissues obtained from the patient and controls at postmortem examinations and stored at −50°C. To prepare an acetone powder, a tissue was minced in at least 15 times its weight of acetone at −50°C and thoroughly homogenized in a Waring Blendor. The suspension was filtered, and the residue was washed successively with acetone and dry, peroxide-free ethyl ether at −50°C. The resulting powder was dried at reduced pressure and stored at 2°C. For extraction, the powder was stirred at 0°C for 2 hours with 0.05*M* potassium phosphate buffer, *p*H 7.8, containing 0.005 percent Versene Fe-3 (*8*), 1 ml of buffer being used for each 100 mg of powder. Sulfite oxidase activity was assayed by determination of the sulfite- and enzyme-dependent reduction of cytochrome *c*. Absorbance changes at 550.5 mμ ($A_{550}$) were measured with a Beckman model DU spectrophotometer. The standard assay mixture, volume 3.1 ml, included: tris-HCl buffer, *p*H 7.6, 100 μmole; cytochrome *c* (horse heart, Type VI, Sigma Chemical Co.), 2.6 mg; $Na_2SO_3$, 10 μmole; Versene Fe-3, 5μg, and enzyme (20 to 50 units). The solution was contained in a cuvette with a 1.0-cm light path and maintained at room temperature (19° to 20°C). The reaction was started by addition of enzyme. The reference solution against which absorbance of the assay mixture was determined contained only tris-HCl buffer and cytochrome *c*. Reaction rate was determined during the period when extent of reaction varied linearly with time, generally between 2 and 12 minutes after enzyme addition. The observed reaction rate was corrected by subtraction of two blank values. One was determined by incubation of enzyme and cytochrome *c* in the absence of added sulfite; the other, determined by omission of enzyme, represented the slow nonenzymatic reduction of cytochrome *c* by sulfite (0.001 absorbance unit per minute). A unit of enzyme activity was considered that amount of enzyme required to catalyze a change of 0.001 absorbance unit per minute under the standard conditions. Protein was measured by the method of Lowry as described by Layne (*9*). All specific activities are expressed as activity per milligram of protein.

Sulfite oxidase activity was readily demonstrable in crude extracts made from acetone powders of human liver. With some extracts a slow rate of enzyme-dependent reduction of cytochrome *c* was observed in the absence of added sulfite. To minimize or prevent this reduction, extracts were routinely purified by passage over a column of Sephadex G-25, maintained at 2°C, equilibrated with tris-HCl buffer, 0.05*M*, *p*H 7.6, containing 0.005 percent Versene Fe-3. Sulfite oxidase was quantitatively recovered after elution with the same buffer without significant change in specific activity.

In tris buffer, the sulfite oxidase activity changed little from *p*H 7.6 to 9.1. The reaction rate at *p*H 6.9 was 60 percent of that at *p*H 7.6. The

Table 1. Sulfite oxidase and other enzyme activities in liver from the patient and control subjects. Sulfite oxidase was measured in Sephadex-treated extracts of acetone powders from livers obtained postmortem. Cystathionase (E.C. 4.2.1.15), methionine-activating enzyme (adenosine triphosphate: L-methionine adenosyltransferase, E.C. 2.5.1.6) and cystathionine synthase were assayed in extracts prepared in phosphate buffer. Standard assay conditions and control values for these enzymes in fresh tissues have been published (*14*, *15*).

| Source of liver | Sulfite oxidase (unit/mg) | Methionine-activating enzyme (mμmole/mg = 60 min) | Cystathionine synthase (mμmole/mg = 135 min) | Cystathionase (mμmole/mg = 30 min) |
|---|---|---|---|---|
| | | *Control subjects* | | |
| Postmortem | | | | |
| No. of patients | 10 | 7 | 8 | |
| Range | 22–87 | 0.3–5.5 | 17–237 | |
| Mean | 51 | 1.5 | 137 | |
| Laparotomy or needle biopsy | | | | |
| No. of patients | | 9 | 9 | 5 |
| Range | | 4.4–9.2 | 133–610 | 6–18.5 |
| Mean | | 6.4 | 252 | 13 |
| | | *Patient T.A.* | | |
| Postmortem | Not detected (<2) | 2.5 | 95 | 14.4* |

* Assayed under modified standard conditions and converted by calculation to units previously defined (*14*).

standard *p*H of 7.6 was selected to minimize the nonsulfite-dependent rate. Replacement of tris buffer by potassium phosphate at *p*H 7.6 led to a 90 percent decrease in rate. Use of equimolar portions of tris and potassium phosphate led to a 73 percent decrease in activity. The rate of cytochrome *c* reduction was proportional to enzyme concentration. Decrease in the cytochrome *c* concentration to one-half the standard concentration led to a 17 percent decrease in rate. Cytochrome *c* at one-fourth the standard concentration yielded a 28 percent decrease in rate. The standard sulfite concentration could be reduced threefold without significant effect on rate. A tenfold decrease led to a barely detectable (about 10 percent) decrease in rate.

Figure 1 illustrates a typical experiment in which extracts from a control subject and from the patient were assayed. The reaction mixture to which control enzyme was added showed an immediate and linear increase in $A_{550}$. This continued until attainment of a plateau value corresponding to complete reduction of cytochrome *c*. The reaction mixture containing control enzyme, but without added sulfite, showed essentially no change. The reaction mixture to which extract from the patient's liver was added showed a very slow decrease in $A_{550}$. At 16 minutes, control enzyme was added to this reaction mixture. After a small immediate shift in $A_{550}$ due to absorption by components of the enzyme solution, there was a progressive, linear rise at a rate equal to that attained with control enzyme alone. This experiment demonstrates that the extract of the acetone powder prepared from the patient's liver contained no detectable sulfite oxidase activity, and that there was no inhibitor or other factor present in this extract which interfered with assay of the control activity. Similar experiments were carried out with both crude and Sephadex-treated enzyme preparations from the patient, over the *p*H range between 6.9 and 9.1. In no case was significant sulfite oxidase activity detected nor was inhibition by the patient's extract observed.

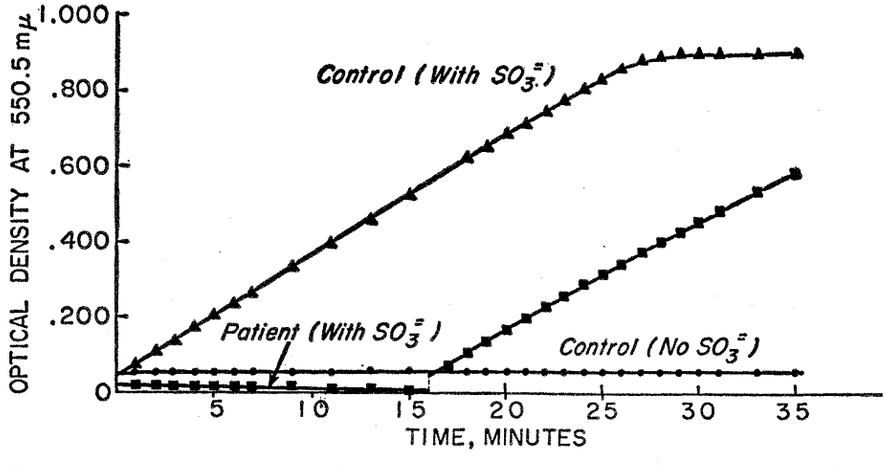


Fig. 1. Sulfite oxidase assays with extracts of acetone powders of liver specimens from the patient and from a control subject. Sodium sulfite was added as indicated. At zero time, extract from a control subject (0.07 ml containing 0.80 mg of protein) or the patient (0.07 ml containing 0.83 mg of protein) was added. At 16 minutes, extract from the control subject was added to the reaction mixture containing the patient's extract. Control experiments in which dithionite was used to reduce the cytochrome *c* demonstrated that the plateau attained with control enzyme at an absorbance of 0.905 was due to complete reduction of the cytochrome *c*.

The results of assays of liver specimens from control patients are summarized in Table 1. The patients had various diseases and ranged in age from 1½ months to 56 years. Postmortem examination was made as long as 14 hours after death. Each control liver had readily detectable sulfite oxidase activity. Activity would have been detected in the patient's liver extract had it been at least 2 unit/mg, a value equal to 4 percent of the mean control value or 10 percent of the lowest control value. The possibility that our failure to detect sulfite oxidase activity in the patient's liver is attributable to a general depression of enzymes in his tissue occurring either before or after death is excluded by several lines of evidence. (i) Tissues were taken from the patient no more than 8 hours after death. Sulfite oxidase activity did not disappear from control postmortem liver during a 14-hour period. There was no apparent tendency for the sulfite oxidase activity of control liver specimens to fall as the time of storage of the tissue at −50°C was increased. The specimens from the patient were stored for a shorter time before extraction than several of the control specimens were. (ii) Three additional enzymes were assayed in extracts prepared from the patient's liver (Table 1). The specific activities of methionine-activating enzyme and cystathionine synthase in the patient's liver were comparable to values determined for control postmortem specimens of human liver and were only slightly depressed even when compared to values for fresh liver (*10*). Cystathionase in the patient's liver was

Table 2. Sulfite oxidase and other enzyme activities in extracts of acetone powders of brain and kidney from the patient and control subjects. All tissues were obtained postmortem. Conditions of the assays were as in Table 1.

| Subject | Sulfite oxidase (unit/mg) | Cystathionine synthase (mμmole/mg = 135 min) | Cystathionase* (mμmole/mg = 30 min) |
|---|---|---|---|
| | *Brain* | | |
| Controls | | | |
| No. of patients | 5 | 5 | 5 |
| Range | 10–13 | 21–38 | 0.03–0.22 |
| Mean | 11.5 | 27 | 0.13 |
| Patient T.A. | Not detected (< 1.5) | 26 | 0.13 |
| | *Kidney* | | |
| Controls | | | |
| No. of patients | 4 | | 3 |
| Range | 22–103 | | 0–5.9 |
| Mean | 63 | | 2.3 |
| Patient T.A. | Not detected (< 1.0) | | 6.7 |

* Assayed under modified standard conditions and converted by calculation to units previously defined (*14*).

within the control range for fresh specimens.

These data prove that the patient's liver had a specific defect in its ability to oxidize sulfite with cytochrome *c* as electron acceptor. The physiological electron acceptor for sulfite oxidase is not known; it may be oxygen (*7*). For this reason, it was conceivable that normal activity of sulfite oxidase would have been detected in the patient's liver had oxygen been used in place of cytochrome *c*, and that the enzyme defect found with cytochrome *c* was fortuitous and without physiological significance. Direct evidence against this possibility was provided by two types of experiments. In one, crude extracts from acetone powders of liver were incubated aerobically with sulfite and without addition of cytochrome *c* or other electron acceptors. Sulfite disappearance was assayed by measurement of sulfite through use of *N*-ethylmaleimide-1-$C^{14}$ (*11*). The patient's liver was less active in catalyzing aerobic sulfite disappearance than control specimens were. In the second experiment, sulfite-$S^{35}$ served as substrate. After aerobic incubation with tissue extracts, reaction mixtures were acidified and gassed with nitrogen to remove unreacted sulfite-$S^{35}$. The radioactive material which did not volatilize under these conditions was taken as a measure of sulfate-$S^{35}$ formation (*12*). With this assay, crude extract of acetone powder from the patient's liver was far less active than extracts made from control livers. Extracts of livers from ten control patients catalyzed the formation of from 0.31 to 1.33 mμmole of sulfate per milligram of protein per minute. The mean value was 0.66 mμmole. The extract from the patient's liver had an activity of 0.09 mμmole per milligram of protein per minute (*13*).

Of mammalian tissues reported to contain sulfite oxidase (*7*), we had specimens of liver, brain, and kidney from the patient (Table 2). For control subjects mean sulfite oxidase activity in brain was one-fifth that in liver. The patient's brain showed no detectable activity; specific activity equal to 13 percent of the mean control or 15 percent of the lowest control value would have been detected. Control values for kidney were comparable to those for liver. The patient's kidney showed no detectable activity. Sensitivity for detection was 2 percent of the mean control and 5 percent of the lowest control value. Recombination experiments similar to those performed with liver showed that the patient's brain and kidney extracts contained no inhibitors or other factors that interfered with determination of sulfite oxidase activity. The extracts of the patient's tissues had specific activities for cystathionine synthase and cystathionase within the range of values obtained with comparable extracts made from control tissues (Table 2). The controls included specimens obtained a longer time after death than the patient's tissues and the control subjects spanned a wide age range.

Our data demonstrate that the patient's liver, kidney, and brain were abnormally low, or entirely deficient, in sulfite oxidase activity. Although we studied only a single case, there can be little doubt that the enzyme defect demonstrated led to the observed chemical abnormalities. We also suggest a causal relation between these abnormalities and the clinical pathology (*2*). The question has been raised whether a compound so readily autoxidizable as sulfite requires an enzyme for its oxidation in the physiological situation (*7*). It has been suggested that this autoxidation, which is a metal-catalyzed, free-radical chain reaction, is ineffective in animal tissues where "chain-breakers" are plentiful and free unbound metal ions may be low in concentration (*7*). Our findings support this suggestion and indicate that enzymatic catalysis by sulfite oxidase is essential in vivo.

S. HARVEY MUDD
*Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, Maryland*

FILADELFO IRREVERRE
*Laboratory of Biophysical Chemistry, National Institute of Arthritis and Metabolic Diseases*

LEONARD LASTER
*Section on Gastroenterology, Metabolic Disease Branch, National Institute of Arthritis and Metabolic Diseases*

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10. As an additional control, cystathionine synthase was measured in the same extracts of acetone powders used for determination of sulfite oxidase activity. The extract from the patient's liver had the highest specific activity of cystathionine synthase of those tested.
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12. Tris-HCl buffer, *p*H 7.6, 7 μmole; $Na_2SO_3$-$S^{35}$, 9 mμmole containing approximately $20 \times 10^3$ count/min, and extract of an acetone powder of liver containing 0.3 to 0.5 mg of protein were incubated in a final volume of 0.15 ml in a test tube of 13 mm (inside diameter) at 26°C for 5 to 20 minutes (in air) with occasional shaking. To terminate the reaction, $Na_2SO_3$, 1 μmole in 1.85 ml of water, was added. The incubation was continued for 6 minutes. After addition of 0.5 ml of a solution containing 2.5 percent $H_3PO_4$, 1.2 percent mannitol, and $5 \times 10^{-3}M$ EDTA, and a drop of capryl alcohol, the mixture was gassed with nitrogen for 1 hour. After dilution of the mixture to 3.0 ml, a portion (0.5 ml) was removed for determination of radioactivity in a Packard Tri-Carb liquid scintillation spectrometer.
13. The slight apparent activity with extract from the patient's liver may not correspond to true sulfate formation. During incubation with pa-

tient's extract nonvolatile radioactive material was formed only during the first few minutes, in contrast to product formation with control enzyme which was proportional to time. Sephadex treatment of the patient's extract largely prevented synthesis of the nonvolatile radioactive material, whereas control enzyme activity was little affected by such treatment.
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16. We thank Dr. I. Fridovich for discussion and the Pathology Department of the Naval Medical Center, Bethesda, Maryland for postmortem-control specimens.

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## L-Asparaginase: Inhibition of Early Mitosis in Regenerating Rat Liver

Abstract. L-*Asparaginase in agouti serum and in extracts from* Escherichia coli *inhibits the early wave of mitosis occurring in rat liver approximately 30 hours after hepatectomy, but even with continued treatment of the animal the later wave at 50 hours is not inhibited. This result differs from the permanent inhibition of growth which asparaginase causes in various tumors.*

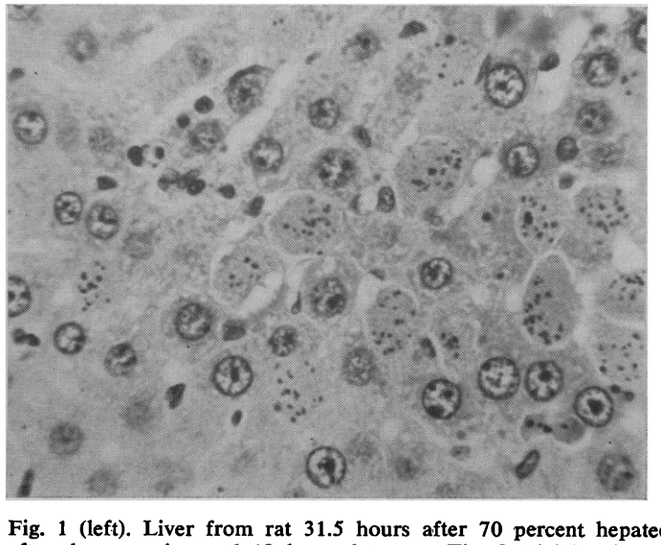
L-Asparaginases of high substrate avidity strongly inhibit the growth of a number of animal tumors of both lymphoid and nonlymphoid origin (*1, 2*). Such enzymes are found in the serums of the guinea pig, agouti, and closely related species, but not in those of other animals (*2, 3*). They are also found in bacteria (*4*). The enzymes produce little general toxicity in treated animals. In earlier studies, animals given asparaginase for prolonged periods maintained normal weights and activity, and no abnormality was found in nonneoplastic tissues upon their inspection with the naked eye or histological examination (*5, 6*). But asparaginase can affect nonneoplastic tissue, as we now show. This enzyme inhibits the early mitosis of the regenerating rat liver to an extent only observed before with toxic amounts of antimetabolites (*7*).

After removal of 70 percent of the liver in the normal rat, an extremely rapid process of growth is initiated, which results in the almost complete restoration of the liver mass within 1 week. The sequence of events by which this takes place has been the subject of considerable study. Following stages of RNA, protein, and DNA synthesis, a wave of mitosis occurs between 25 and 36 hours after hepatectomy, and a later, smaller wave takes place between 47 and 53 hours (*8*). We have examined the effect of asparaginase on liver mitosis using enzyme from two sources: agouti serum, obtained by cardiac puncture of mature animals (*2*), and *Escherichia coli* extract, purified by salt fractionation and chromatography essentially by the method of Mashburn and Wriston (*4*).

Hepatectomy or sham operation was performed on male Sprague-Dawley rats (250 ± 25 g) as described previously (*9*). Immediately afterwards, asparaginase preparations or control solutions were injected intravenously. Further injections of asparaginase or other solutions were given at 12-hour intervals. Rats were killed 31.5 or 52.5 hours after the operation, in each case 6 hours after the intraperitoneal injection of colchicine (1.0 mg/kg) given to arrest mitoses. Livers and other organs were fixed in 10 percent formalin, sectioned at 5 $\mu$, and stained with hematoxylin and eosin. The number of mitoses in 12,000 nuclei were counted in each liver; a notable uniformity was found between counts from sections of different lobes of the organ.

The numerous mitoses of the early wave are obvious in a control rat killed 31.5 hours after hepatectomy (Fig. 1). The scattered distribution of the chromosomes is the result of treatment with colchicine. By contrast, in a similar rat treated with 2.0 ml of agouti serum [810 units of asparaginase assayed according to Broome (*10*)] at the time of hepatectomy and 12 hours later, no evidence of mitotic activity is visible. Nonetheless, the hepatic cells do not appear abnormal so far as can be observed with light microscopy. The nonregenerating liver contains few mitoses (Table 1); 31.5 hours after hepatectomy the mitotic index has risen to 5.4 percent. At this time in hepatectomized animals given asparaginase either in agouti serum or in the preparation from *E. coli* almost no mitoses can be counted. The total quantities of asparaginase given are not large compared to those that inhibit the growth of rat tumors (*5, 11*).

Normal rabbit serum and a crude preparation of *E. coli* glutaminase, both of which lack asparaginase activity, fail to inhibit mitosis. In other experiments



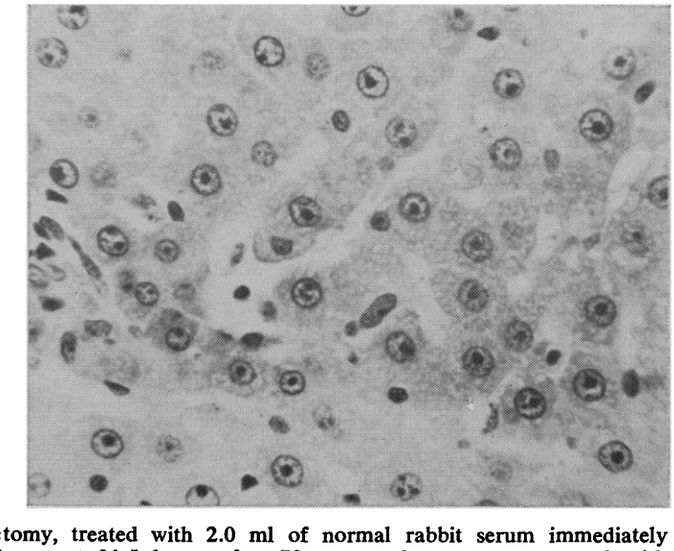


Fig. 1 (left). Liver from rat 31.5 hours after 70 percent hepatectomy, treated with 2.0 ml of normal rabbit serum immediately after the operation and 12 hours later. Fig. 2 (right). Liver from rat 31.5 hours after 70 percent hepatectomy, treated with agouti serum as described in the text (× 450).