Correction. In the article "Conformation of uncomplexed natural antamanide crystallized from CH$_3$CN/H$_2$O" by Isabella L. Karle, T. Wieland, D. Schermer, and H. C. Ottenheym, which appeared in the April 1979 issue of Proc. Natl. Acad. Sci. USA (76, 1532–1536), Figs. 1, 3, and 4 were not printed well. The figures and their legends are reprinted here.

![FIG. 1](image1)

**FIG. 1.** Stereodiagram of natural antamanide drawn by computer from experimentally determined coordinates. The peptide backbone is outlined by heavy bonds and the C" atoms are labeled 1 to 10. Four H$_2$O molecules are indicated by Roman numerals.

![FIG. 2](image2)

Correction. In the article "Growth and morphology of colonies of Chinese hamster ovary cells growing on agar is affected by insulin" by Bruce D. Aidells, Michael W. Konrad, and Donald A. Glaser, which appeared in the April 1979 issue of Proc. Natl. Acad. Sci. USA (76, 1863–1867), Fig. 4 failed to show all the details of the original. The figure and its legend are reproduced below.

![FIG. 3](image3)

**FIG. 3.** Channel formation in the crystal of [Phe$^4$,Val$^6$]antamanide. Three water molecules closely associated with each peptide molecule are indicated by darkened circles. The axial directions are $c$ ↑, $b$ ←, and $a$ directed up from the page.

![FIG. 4](image4)

**FIG. 4.** Stereodiagram of the packing in the crystal of natural antamanide. Water molecules are indicated by darkened circles and hydrogen bonds are denoted by light lines. The axial directions are $a$ ↑, $b$ ←, and $c$ directed up from the page.

Correction. In the article "Repair of O$^6$-methylguanine in adapted Escherichia coli" by Paul F. Schendel and Peter E. Robins, which appeared in the December 1978 issue of Proc. Natl. Acad. Sci. USA (75, 6017–6020), the authors request the following change. The units given for the left vertical axis of Fig. 2 should be cpm in O$^6$MeGua/3 × 10$^7$ cells.

Correction. In the article "Modification of the blood–brain barrier: Increased concentration and fate of enzymes entering the brain" by John A. Barranger, Stanley I. Rapoport, Wendy R. Fredericks, Peter G. Pentchev, Kaye D. MacDermot, Jan K. Steusing, and Roseoe O. Brady, which appeared in the January 1979 issue of the Proc. Natl. Acad. Sci. USA (76, 481–485), the authors wish the following correction to be made. On page 485, line 1 of the left-hand column, ref. 18 should be deleted and added on page 484 to the end of line 8 from the bottom of the right-hand column so that the references in these parentheses will be "(18, 20)."

Fig. 4. Determination of mounding index. By dividing the radius of each colony into thirds, we can quantitate morphological differ-
Modification of the blood–brain barrier: Increased concentration and fate of enzymes entering the brain

(arabinose/mannosidase/horseradish peroxidase/lysosomes)

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Contributed by Roscoe O. Brady, October 26, 1978

ABSTRACT  The blood–brain barrier of rats was opened reversibly by infusing a hyperosmotic solution of arabinose into the external carotid artery. Permeability was increased maximally in the first 15 min and remained slightly elevated at 1 hr. Osmotic barrier opening significantly increased brain uptake of intravenously injected α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.2,4) (derived from human placenta) and horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7). By injection of 4 x 10⁵ units of α-mannosidase into an animal, brain activity rose to about twice the normal control activity of the enzyme. After 30 min, activity of administered enzyme in the extracellular space of the brain was calculated to be 30% of the serum concentration. Biochemical and histological studies with horseradish peroxidase showed that exogenously administered enzyme entered the brain extracellularly and immediately after barrier opening and was incorporated within neuronal lysosomal packets during the next 24 hr. Measureable peroxidase activity was found in brain as much as 72 hr after osmotic treatment. The results demonstrate that the blood–brain barrier can be reversibly opened to enzymes, that a glycoprotein enzyme is incorporated into neuronal lysosomes, and that the brain may now be considered a potential target for enzyme replacement therapy in heritable metabolic disorders.

The blood–brain barrier at cerebral blood vessels is due to a continuous layer of endothelial cells bound together by tight junctions. The cells show little or no transendothelial vesicular transport. Because tight junctions prevent intercellular diffusion, the barrier restricts brain–blood exchange of water-soluble drugs, ions, and proteins, although lipid-soluble agents easily enter the brain (1–3).

The blood–brain barrier can be altered experimentally by a number of different methods (1, 4). One of these is osmotic barrier opening, which, unlike most other methods, is reversible and is unaccompanied by evidence of gross neurological injury (5, 6). Osmotic barrier opening most likely is caused by shrinkage of cerebrovascular endothelial cells and increased permeability of interendothelial tight junctions (ref. 7 and unpublished data). Permeability is increased to the Evans blue dye–albumin complex (M, 78,000) and to neutralizing antibody (IgG) to measles virus (M, 180,000). Antibody that is allowed into the brain retains activity for up to 4 days (8).

The observation that the blood–brain barrier can be opened reversibly in laboratory animals without gross injury led us to consider introducing an intravascularly administered lysosomal enzyme into the brain and to examine its cerebral disposition in an intact animal. This report presents the results of these studies and describes the intensity and time course of barrier modification. Some of the work has been reported in preliminary form (9).

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MATERIALS AND METHODS

Materials. Horseradish peroxidase type II (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) and (l(+)-arabinose were purchased from Sigma. DEAE-cellulose was obtained from Whatman. Concanavalin A-Sepharose was obtained from Pharmacia. 3,3'-Diaminobenzidine was a product of Baker. 4-Methylumbelliferyl acid hydrolase substrates were obtained from Koch-Light, (Colnbroon, England). Evans blue was from Chroma-Gesellschaft, (Stuttgart, Germany). [14C]Sucrose, [14C]mannitol, and [14C]dextran were obtained from New England Nuclear. All other chemicals were reagent grade. Fresh human placentas were obtained from local hospitals and stored in 0.3% streptomycin sulfate at 4°C until used.

Modification of Blood–Brain Barrier. Male Sprague–Dawley rats weighing 250–350 g were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The skin of the neck was incised and the external carotid artery was exposed and freed of connective tissue. A polyethylene catheter (PE 50), filled with 0.9% (wt/vol) NaCl plus 100 international units of Na heparin per ml, was tied into the external carotid artery for retrograde infusion. The mouth of the catheter was placed 2 mm from the bifurcation of the common carotid. A solution of 1.6 molal arabinose, warmed to 37°C, was infused through the catheter for 30 sec, at a rate of 0.12 ml/sec, by means of a constant flow pump. Direct observation of the carotid artery tree revealed that infusate passed into the brain via the internal carotid artery without flowing down into the common carotid.

Evans blue dye–albumin (0.5 ml of 2% solution) was given intravenously 5 min prior to carotid infusion. Entry of dye into the brain ipsilateral to carotid infusion served as a positive marker of barrier alteration, which was graded from grade 0 (no brain staining by Evans blue) to 3+ (deep blue staining). Only animals with a grade 2+ or 3+ staining, comprising 85% of experiments, were used for further analysis.

Time Course of Barrier Modification. Previous studies qualitatively demonstrated that osmotic barrier opening to intravascular Evans blue–albumin was reversible. In the present study, we used a recently reported method (10) to quantitatively determine the time course of increased cerebrovascular permeability after 1.6 m arabinose infusion. Five microcuries of [14C]Sucrose (specific activity 4.9 Ci/mol) was injected intravenously at the following times after carotid infusion: 5 min, 30 min, 60 min, 120 min, 1 day, and 2 days. Plasma radioactivity was followed for 10 min after tracer was injected. When the animal was killed, three regions of the ipsilateral brain were dissected and prepared for measurement of radioactivity.

Preparation of α-Mannosidase. α-Mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) was prepared from human placenta by using columns containing concanavalin A
and DEAE to enrich the enzyme 1000-fold over the starting concentration (unpublished observations). Further purification to a specific activity of \(2.4 \times 10^6\) units/mg of protein could be accomplished, but the product was cleared so rapidly from blood that it was not useful for the present experiments. Thus, enzyme with a specific activity of \(3.0-6.0 \times 10^4\) units/mg of protein was used routinely.

Changes in Brain Concentration and Disposition of \(\alpha\)-Mannosidase or Peroxidase. Animals were prepared as described above. Approximately 1 min before carotid infusion of arabinose, either \(4-5 \times 10^6\) units of \(\alpha\)-mannosidase or 20 mg of peroxidase was injected intravenously as a bolus. Blood samples were removed from a femoral artery catheter every 5 min for 1 hr, then every 10 min for an additional hr. Animals were killed 2 hr after carotid infusion in the initial experiments. Brains were removed to ice-cold 0.25 M sucrose containing 5 mM Tris-HCl, 1 mM EDTA, and 0.1% Triton X-100. Ten percent homogenates were prepared for assay of enzymatic activity.

Enzyme Assays. \(\alpha\)-Mannosidase, \(\beta\)-galactosidase, and acid phosphatase were assayed with 1 mM solutions of 4-methylumbelliferyl pyranosides or the respective phosphate in citrate/phosphate buffer (pH 4.5) containing 5 mg of bovine serum albumin per ml. Reaction volume was 100 \(\mu\)l. Units are expressed as nmol hydrolyzed per hr at 37°C.

Peroxidase was assayed as described (11). Glucocerebrosidase was measured as reported by Furbish et al. (12). Lactate dehydrogenase was measured by the method of Amador et al. (13). Cytochrome c succinic reductase was assayed by the method of Tisdale (14).

Subcellular Fractionation. Subcellular fractions of brain were prepared by a modification of the method of DeDuve et al. (15). Brain was homogenized in 4.5 vol of ice-cold 0.25 M sucrose by using two passes of a Teflon pestle in a smooth glass tube with a 0.01-inch (0.25-mm) clearance. This was repeated after an additional 4.5 vol of sucrose was added. The homogenate was centrifuged at 600 \(\times\) g for 10 min. The supernatant was removed and the pellet was washed once with buffer and centrifuged again. The supernatant was combined with the previous supernatant and centrifuged at 25,000 \(\times\) g for 10 min. The pellet was separated from the supernatant, which was centrifuged at 100,000 \(\times\) g for 30 min. The pellets were designated nuclear (N), mitochondrial–lysosomal (Mr–L), or microsomal (M). The supernatant was designated as the soluble fraction (S).

Histochemical Procedure for Peroxidase. The method of Graham and Karnovsky (16) was used except that the tissue was washed for 2 hr.

RESULTS

Time Course of Barrier Modification. Previous studies showed that the hemibrain ipsilateral to carotid infusion becomes stained blue by entry of dye, and this staining correlates with permeability changes to \[^3H\]norepinephrine and \[^125I\]-

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Table 1. Augmentation of brain \(\alpha\)-mannosidase by intravenous infusion of enzyme and blood–brain barrier modification

<table>
<thead>
<tr>
<th>Carotid infusion</th>
<th>Enzyme infusion, units/kg</th>
<th>Grade of staining</th>
<th>Enzyme content, units/g</th>
<th>Right hemibrain</th>
<th>Left hemibrain</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0</td>
<td>3150</td>
<td>3300</td>
<td></td>
</tr>
<tr>
<td>Arabinose, 1.6 molal</td>
<td>None</td>
<td>2+</td>
<td>3010</td>
<td>3200</td>
<td></td>
</tr>
<tr>
<td>Arabinose, 1.0 molal</td>
<td>(5 \times 10^5)</td>
<td>0</td>
<td>3250</td>
<td>3200</td>
<td></td>
</tr>
<tr>
<td>Arabinose, 1.6 molal</td>
<td>(5 \times 10^5)</td>
<td>2+</td>
<td>5300</td>
<td>3300</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2+</td>
<td>5000</td>
<td>3000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2+</td>
<td>6200</td>
<td>3400</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2+</td>
<td>4600</td>
<td>3200</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2+</td>
<td>5900</td>
<td>3550</td>
<td></td>
</tr>
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<td></td>
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<td>2+</td>
<td>6250</td>
<td>3050</td>
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<tr>
<td></td>
<td></td>
<td>2+</td>
<td>4200</td>
<td>3600</td>
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<tr>
<td></td>
<td></td>
<td>2+</td>
<td>5450</td>
<td>3800</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>5362</td>
<td>3238 Δ2124</td>
<td></td>
</tr>
</tbody>
</table>

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Animals were infused with arabinose through the right carotid artery and killed after 2 hr. Brains were removed and placed in an ice-cold buffer containing 5 mM Tris-HCl, 1 mM EDTA, and 0.25 M sucrose at pH 7.5. The meninges were stripped away and the brains were divided into left and right halves and enzymatic activity was determined.

* 1.0 molal arabinose is a subthreshold concentration of osmotic agent needed to produce barrier modification.
labeled albumin (17). Cerebral capillary permeability was measured in different regions of the brain with $^{14}$C)sucrose and it was shown to be independent of possible changes in regional blood flow (10). This method was used to determine the time course of increased cerebrovascular permeability in four brain regions after carotid infusion of 1.6 molal arabinose. Fig. 1 illustrates that permeability increased maximally (by about 20-fold over control) in three regions supplied by the internal carotid artery (the caudate nucleus, hypothalamus, and frontal lobe) and then declined rapidly. The pons, supplied by the vertebral circulation, was spared. Little alteration of the barrier was present at 2 hr. The findings agree with studies of reversibility using the qualitative Evans blue–albumin tracer and demonstrate that the maximum increase in permeability after osmotic treatment is short-lived.

**Augmentation of α-Mannosidase in Rat Brain.** Eight rats were injected intravenously with α-mannosidase. Serum enzyme activity was constant for 30 min and ranged between 35,000 and 40,000 units/ml. It then declined with a subsequent half-life of about 10 min. After 2 hr, serum activity was approximately 900 units/ml. The barrier was unmodified in the unperfused contralateral hemibrain and, therefore, each animal served as its own control. The net quantity of enzyme that entered the perfused hemibrain is the difference between the perfused and unperfused hemibrain concentrations. The enzyme in the contralateral control hemibrain is made up of the endogenous enzyme in brain tissue and the exogenous enzyme in the cerebrovascular space. However, intravascular enzyme activity was negligible at 2 hr. Since blood constitutes not more than 3% of brain wet weight (10), and since the 2-hr serum enzyme activity equaled 900 units/ml, total intravascular activity at 2 hr would be about 27 units/g of brain, as compared to background of approximately 3000 units/g in brain tissue itself. This activity in the control contralateral hemibrain in the animals that were treated osmotically and infused with α-mannosidase was the same as in animals not receiving enzyme. The average increase in α-mannosidase activity after hypertonic infusion was 2100 units/g of brain for a dose of $5 \times 10^6$ units of enzyme per animal (Table 1). Permeability was significantly altered for only the first 30 min after carotid infusion (Fig. 1). During this time, the serum concentration of enzyme was essentially constant at 35,000 units/ml. Data presented below indicate that the enzyme initially was found in the brain extracellular space, which constitutes about 20% of wet weight of the brain (1, 18). Taking these points into account, the brain space into which the enzyme distributed can be calculated as 6% of brain weight (2100 units/g of brain)/(35,000 units/ml of serum); the fraction of extracellular space filled by enzyme can then be calculated as 30% or 6/20.

**Disposition of Exogenous Enzyme within Brain.** In order to eliminate possible errors due to endogenous enzyme activity, we used peroxidase to study intracerebral disposition and cellular uptake of enzymes. The blood–brain barrier was opened by infusion of 1.6 molal arabinose in the right carotid artery, and peroxidase was injected intravenously as a bolus. Two hours later, enzyme activity was readily demonstrated in homogenates of the ipsilateral, but not in the contralateral, hemibrain (Table 2). Centrifugation at 100,000 $\times g$ for 30 min showed that most of the peroxidase was in the supernatant fraction 2 hr after hypertonic arabinose infusion. Animals killed at later times had measurable peroxidase activity in the particulate fraction. The amount of activity associated with the particulate fraction increased with time up to 72 hr (Fig. 2). By 24 hr, over 90% of recovered activity was associated with the 100,000 $\times g$ pellet. Addition of 500–5000 units of peroxidase to homogenates of 1 g of control brain tissue did not result in more than trace amounts of activity associated with the particulate fraction. Similar results were obtained when $^{14}$C]mannitol or $[^{14}$C]dextran was homogenized with control hemibrain. These findings indicate that the peroxidase associated with the particulate fraction was incorporated into an organized particle within brain cells.

Histochemistry of cortical sections of perfused hemibrain supported this interpretation (Fig. 3). Dense peroxidase staining of granular material was evident in the cytoplasm and axonal projections of cerebral cortical neurons. The nuclei apparently did not stain. No cells were stained in the contralateral cortex, where the barrier remained intact. Staining, which was evident in the right cerebral cortex of animals killed 72 hr after hypertonic infusion, was qualitatively less than at 48 hr, which in turn was less than at 24 hr. Intercellular staining was marked at 2 hr and barely visible at 24 hr. Thus, peroxidase was slowly disposed of by the brain.

Differential centrifugation of brain homogenates showed that
peroxidase activity was associated with the particulate fraction containing lysosomal enzyme markers (Fig. 4) and, to some extent, with the nuclear fraction. A small amount of lactate dehydrogenase was associated with this nuclear fraction, which light microscopy demonstrated to be devoid of whole cells. Preliminary electron microscopic examination revealed membrane-bound, peroxidase-positive particles in both the nuclear and lysosomal fractions (unpublished observations). The recovered activity from the nuclear and lysosomal fractions showed latency; i.e., total activity could be measured only after treatment by techniques that disrupt lysosomal membranes.

**DISCUSSION**

We have demonstrated that the blood–brain barrier can be made permeable to enzymes in a predictable and reversible manner by carotid infusion of 1.6 molal arabinose solution. The findings are in agreement with less complete observations in which hypertonic mannitol solution was used to alter the barrier (9). Permeability is increased maximally in the immediate 15 min after hypertonic infusion and remains slightly elevated for up to 2 hr, as reported earlier (19).

Although brain activity of α-mannosidase was elevated to 166% of the endogenous level in these experiments by injecting 4–5 × 10⁶ units of α-mannosidase, brain uptake might be increased much more by infusing the enzyme into the external carotid artery catheter rather than by injecting it intravenously. Experiments using [³H]methotrexate as a barrier opening tracer demonstrate that carotid administration increases the amount of drug entering barrier-altered brain by a factor of 5 above that produced by systemic intravenous infusion (unpublished observations). A combination of osmotic barrier opening and carotid infusion could, in principle, increase brain enzyme concentrations many times above the low endogenous levels found in many genetic disorders. Normal enzyme concentrations should be readily achievable by this method, at least temporarily.

The experiments with peroxidase show that enzyme that enters the brain is initially restricted to the extracellular space. The enzyme can be eliminated from this space by diffusing into cerebral spinal fluid or back across capillaries into blood (20) or by inactivation. However, we have shown that a significant quantity is incorporated into brain cells and is localized within intracellular lysosomes. The retention of intracerebral enzymatic activity for up to 72 hr indicates that the enzyme is not rapidly degraded or otherwise inactivated within lysosomes.

Other studies have demonstrated peroxidase uptake by neurons into granular and membrane-bound particles that
probably are lysosomes (18, 19, 21–23). Systemically administered peroxidase can be taken up in membrane-bound vesicles at peripheral nerve endings, which then are transported in a retrograde direction into central nerve-cell bodies of cranial nerves (19). As the peroxidase-laden particles enter the axon hillock, they become positive in acid phosphatase, whereas they are not positive in the peripheral nerves (24). Our results show that enzyme entering the brain after osmotic barrier opening can be incorporated into particles of neuronal cell bodies and their processes. Studies with intracerebral injection of peroxidase have demonstrated uptake at axon terminals and retrograde transport to cell bodies (21). Whether the appearance of peroxidase in neurons proceeds by direct uptake into cell bodies or by retrograde transport or both is not addressed by our experiments.

The apparent lack of peroxidase staining in glial elements is provocative. This could represent rapid disposition of the enzyme by glial cells such that sampling at 2 hr or later might not reveal their involvement. On the other hand, the absence of peroxidase activity in glia could indicate a specific process in neurons. Peroxidase is a glycoprotein rich in mannosyl residues. Specific receptors for mannose-terminal glycoproteins have been demonstrated in cells of non-neural tissue (25, 26). The possibility exists that a similar mannosyl-specific receptor is present on neuronal membranes.

The appearance of peroxidase in the crude nuclear fraction has been seen before in experiments attempting to follow the fate of intravenously administered enzymes (27–29), but no satisfying explanation for it is evident. One possibility is that so-called "endocytotic vacuoles" or complete lysosomes coalesce into particles whose specific gravity equals that of nuclei. The observation that this fraction, as well as the lysosomal fraction, demonstrate latency supports this hypothesis.

In summary, we have shown that a significant amount of a human lysosomal enzyme enters the brain after osmotic barrier opening, and we have suggested that this amount may be further augmented by administering the enzyme via an intracarotid rather than an intravenous route. Increased enzymatic activity remains in the brain for up to 72 hr and is localized within neuronal lysosomes. These findings provide encouragement for the possibility of enzyme replacement therapy in inborn deficiencies of lysosomal enzymes involving the brain.