the naphthol AS-BI derivatives of both β-D-*N*-acetylglucosamine and β-D-*N*-acetylgalactosamine as substrates, as well as 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide. The activity of hexosaminidase component B was markedly increased in cerebral cortex from the Tay-Sachs patients compared to controls (Table 2), explaining the high levels of hexosaminidase activity in this tissue (Table 1), but hexosaminidase A was not detected.

In three other neuronal lipid storage disorders, generalized gangliosidosis, Hurler's syndrome, and late infantile amaurotic idiocy, no deficiency of hexosaminidase component A was found (Fig. 1). These results indicated that ganglioside storage in itself, neuronal lipidosis, slowly progressive fatal cerebral degeneration, and prolonged storage of frozen tissues were not responsible for the deficiency of hexosaminidase component A in the brain of Tay-Sachs patients.

It seemed unlikely that storage of ganglioside GM_2 in itself accounted for the deficiency of hexosaminidase A, since the deficiency was the same in brain, where ganglioside storage is massive, and in liver, kidney, and skin, where ganglioside storage is minimal.

When brain or liver homogenates from controls and Tay-Sachs patients were mixed in equal proportions, the activity of hexosaminidase component A in the mixed sample was the average of the control and Tay-Sachs activities. This finding indicated that soluble endogenous inhibitors (including ganglioside GM_2 which was soluble in the buffers used) were not responsible for the complete inactivity of component A.

Starch-gel electrophoretic studies of other glycohydrolases from cerebral gray matter and liver tissues of the Tay-Sachs patients revealed no alteration of enzyme patterns. This was true of β-galactosidase, which can be separated into three or four components in gray matter and in liver (*14*), as well as β-glucuronidase and β-glucosidase, which can be separated into two or three components (*15*).

Studies on fresh venous blood demonstrated that component A was the major hexosaminidase in normal human plasma and only traces of component B were present. Component A was absent in fresh plasma obtained from two living patients with Tay-Sachs disease; all the hexosaminidase activity was due to component B.

Leukocytes from control subjects contained both hexosaminidase components in the ratio of 73 percent A to 27 percent B. Leukocytes obtained fresh and assayed immediately from both living patients with Tay-Sachs disease contained component B; component A was absent (Fig. 1). Hexosaminidase A was absent in cultured skin fibroblasts (greater than ten cellular generations) obtained from three patients with Tay-Sachs disease; skin fibroblasts cultured from control subjects contained high concentrations of hexosaminidase A. Tay-Sachs fibroblasts contained normal concentrations of hexosaminidase B.

The absence of a β-D-*N*-acetylhexosaminidase component which possesses both *N*-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase activity could provide a satisfactory explanation for the ganglioside storage in Tay-Sachs disease. A block in the catabolism of ganglioside GM_2, which contains a terminal *N*-acetylgalactosamine residue, could result from the absence of such a hydrolytic lysosomally localized enzyme. Before acceptance of this explanation, it must be demonstrated that hexosaminidase component A participates in the catabolism of ganglioside GM_2. Nonetheless, the fact that the deficiency occurs in all Tay-Sachs tissues studied and that levels of hexosaminidase A in plasma and leukocytes in heterozygous carriers of the Tay-Sachs gene are intermediate between homozygous affected patients and controls (*15*) suggests that the enzyme deficiency is closely related to the genetic defect.

The immediate practical importance of our discovery is that hexosaminidase assay provides a means for the diagnosis of homozygotes. We have found (*15*) that both hexosaminidase components are present in normal fetal amniotic fluid cells obtained by amniocentesis early in pregnancy. If component A is absent in fetal amniotic cells derived from individuals homozygous for Tay-Sachs disease, as appears likely, the intrauterine diagnosis of this fatal human disease will be possible.

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Energized Configurations of Heart Mitochondria in situ

Abstract. *Changes in the configurational state of the cristal membranes of rat heart mitochondria within the living cell can be induced by imposing energizing conditions. An exact correlation has been established between the configurational states of the cristal membrane and the energy states of the mitochondrion. The configurational changes observed in mitochondria* in situ *are comparable to those established for beef heart mitochondria in vitro and are consistent with the postulate of the conformational basis of energy transductions in membrane systems. The formation of paracrystalline arrays is one of the noteworthy features of configurational changes of mitochondria* in situ.

In earlier communications (*1, 2*) a correlation has been established between the configurational state (*3*) of cristal membrane (as determined by electron microscopy or by light scattering) and the energy state (*4*) of the beef heart mitochondrion in vitro. The extensive configurational changes that have been induced by experimental means (*5*) in isolated mitochondria did

not support the evidence which demonstrated that mitochondria *in situ* are found predominantly in what is called an orthodox configuration. Only in some instances have mitochondria fixed *in situ* been shown to have alternative configurational states (*6*). The contrast between the configurational lability of isolated mitochondria under controlled experimental conditions and the configurational stability of mitochondria *in situ* has naturally engendered some skepticism concerning the significance of these extensive configurational changes. Occasionally, investigators who took special precautions in fixing tissues succeeded in recognizing extensive configurational changes of mitochondria *in situ* (*6*). However, because no control was exercised over the energy state of mitochondria, these observations have had limited value as supportive evidence of our thesis that the configurational state of the mitochondrion is an expression of the energy state.

We have developed methods that have enabled us to demonstrate, consistently and quantitatively, configurational transitions in mitochondria *in situ*. We present here evidence of configurational changes in rat heart mitochondria under controlled experimental conditions.

Albino rats which had been maintained on laboratory chow were immobilized by cervical dislocation. The beating hearts were rapidly removed and placed in ice-cold, Ca^{++}-free, modified Krebs-Ringer-phosphate (KRP) medium (*7*). The ventricles were diced with a razor blade into pieces the size of pinheads in a pool of ice-cold KRP on a piece of hard dental wax. Several pieces of the tissue were immediately transferred to the incubation medium. The oxygen tension was maintained at a level which insured the aerobic state of the tissue by continuously bubbling the incubation medium with oxygen. After 2 minutes of incubation, the suspension of diced tissue was fixed for electron microscopy by the addition of an equal volume of ice-cold KRP (2 percent in formaldehyde and 2 percent in glutaraldehyde). The tissue was fixed for 90 minutes at 3°C in the formaldehyde-glutaraldehyde mixture. The excess fixative was washed away with KRP, and the tissue was fixed with 2 percent OsO_4 in KRP for 90 minutes at 3°C. The specimens were then dehydrated in a graded series of ethanol, dry propylene oxide, and then embedded in Epon (*8*). Thin sections were cut by glass or diamond knives with a Porter-Blum ultramicrotome model MT-2. The sections were mounted on carbon-covered grids, stained with lead citrate, and examined in a Hitachi HU-11B electron microscope at 75 kv.

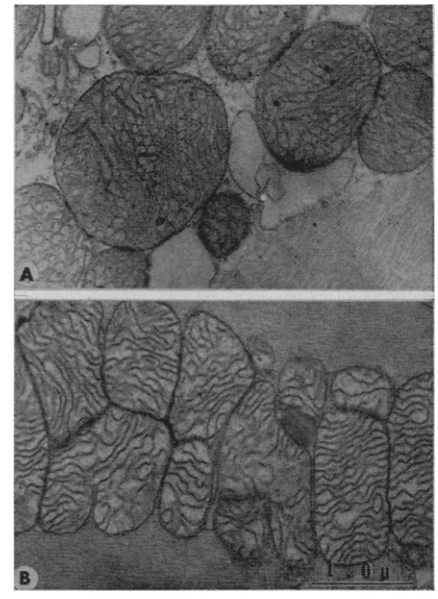

Fig. 1. The basic incubation medium was the modified KRP solution (6 ml) which contained rotenone (17 μg/ml), rutamycin (67 μg/ml), and sodium iodoacetate (1 mmole/liter). The samples were incubated for 2 minutes in a water bath at 20°C. (A) Mitochondria in the energized configurations observed in the presence of 10 m*M* sodium succinate (state 4 respiration conditions); (B) mitochondria in the nonenergized configuration obtained in the presence of 10 m*M* sodium succinate and 0.4 m*M* dinitrophenol (state 3 uncoupled respiration).

Under energizing conditions the entire mitochondrial population in the heart tissue assumed the energized or energized-twisted configuration (Fig. 1A); however, under precisely the same experimental conditions, except for the presence of 2,4-dinitrophenol—a reagent which discharges the energized state of the cristal membrane—the cristal membranes are in the nonenergized configuration (Fig. 1B). We have repeatedly found an invariant correlation between the configurational state and the energy state of rat heart mitochondria *in situ* (*9*). When no precautions were taken to impose energizing conditions, the mitochondrial population was found to be predominantly in the nonenergized configuration, as if dinitrophenol were present.

Data from electron micrographs have made it possible to specify each of the three configurational states of the cristae of rat heart mitochondria *in situ* (Fig. 2). The state induced by dinitrophenol is the nonenergized configuration. This is the state in which mitochondria are usually found when tissues are processed by conventional methods. The zigzag state (see Figs. 1A and 4B) can be identified as the energized-twisted configuration because precisely the same configurational state can be induced in mitochondria in vitro only when both substrate and inorganic phosphate are present in the medium. The energized configuration can also be identified on the basis of the analogy with the corresponding state defined experimentally in mitochondria in vitro (*1, 2*). Therefore, under energizing conditions all the cristal membranes are either in the energized or energized-twisted configurations, whereas under nonenergizing conditions (in presence of uncoupler or under anaerobiosis) all the cristal membranes are in the nonenergized configurations.

The cristal membranes of beef heart mitochondria in vitro (*1, 2*) are usually found in the aggregated mode, whereas the cristal membranes of mitochondria *in situ* are usually found in the orthodox mode. In the aggregated mode, the

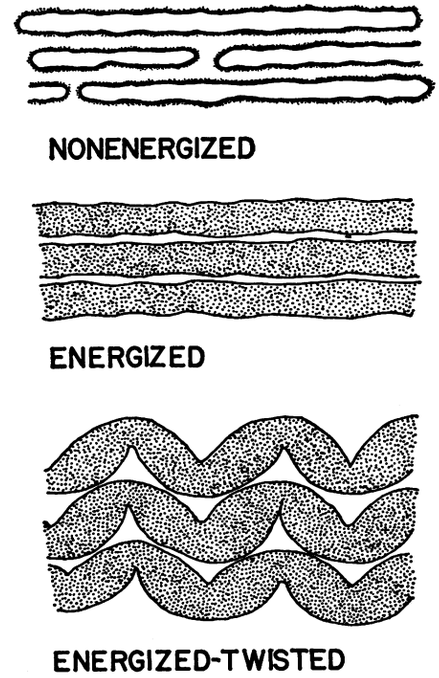

Fig. 2. Three configurational states of the cristal membrane of rat heart mitochondria *in situ*.

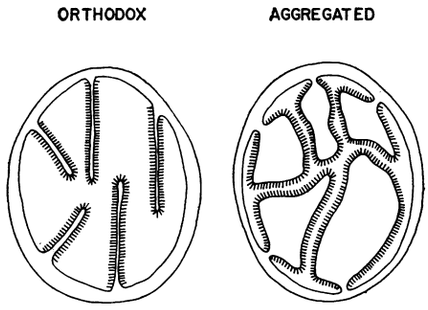

Fig. 3. Aggregated and orthodox modalities of the cristal membrane. This is an attempt to represent, in principle only, the major difference between the aggregated and orthodox modalities. [Original drawing provided by Dr. E. F. Korman]

cristal membranes pack with one another by close apposition of the faces of the membranes which have headpiece-stalk sectors. This makes the matrix space electron-opaque (see Fig. 3). In the orthodox mode, the cristal membranes are much farther apart and thus present an electron-transparent matrix space, resulting in the light regions in Fig. 1B. It is experimentally possible to induce transitions from the aggregated to the orthodox mode with isolated mitochondria in vitro (*2, 5, 10*). The electron micrographs of Fig. 1 show that the nonenergized configuration of the cristal membrane of rat heart mitochondria *in situ* is in the orthodox mode. The energized cristal membranes are in the aggregate mode. It appears, therefore, that the in vivo transition from the orthodox to the aggregated mode of the crista is intrinsic to the energy cycle. Figure 4A is an electron micrograph of a mitochondrion in transition from the energized configurations of the aggregated mode (right side) to the nonenergized configuration of the orthodox mode (left side).

When the cristal membranes become energized they often pack together in a regular fashion to form paracrystalline arrays (*11*) (Fig. 4, B, C, and D). "Stars of David" in Fig. 4D are a particularly interesting example arising by viewing closely packed, tubular cristae in a section perpendicular to the long axis of the cristae. Also there is evidence that paracrystalline arrays observed widely in mitochondria are invariably produced by the way adjacent cristae fold, stack, aggregate, or expand either in the energized or nonenergized configurations (*12*).

The following procedures appear to be essential for demonstrating consistently configurational states of mitochondria *in situ*. (i) The tissue was rapidly removed from the animal and finely subdivided without extensive injury to the cells. (ii) Energizing conditions were imposed upon the tissue by bubbling oxygen into a medium containing substrate for electron transfer. (iii) Inhibitors were present which prevented the discharge of the energized state (rotenone and rutamycin). (iv) The incubated suspension was rapidly fixed (formaldehyde plus glutaraldehyde) before anaerobiosis could occur.

Although we focused our attention in this report upon documentation of the correlation between energy state and configurational state of rat heart mitochondria *in situ*, we have in fact carried out many similar experiments with other tissues, for example, rat liver, rat kidney, ox retina, canary heart and canary flight muscle. All such experiments have proven to be confirmatory and completely consistent with the results reported herein with rat heart muscle (*13*).

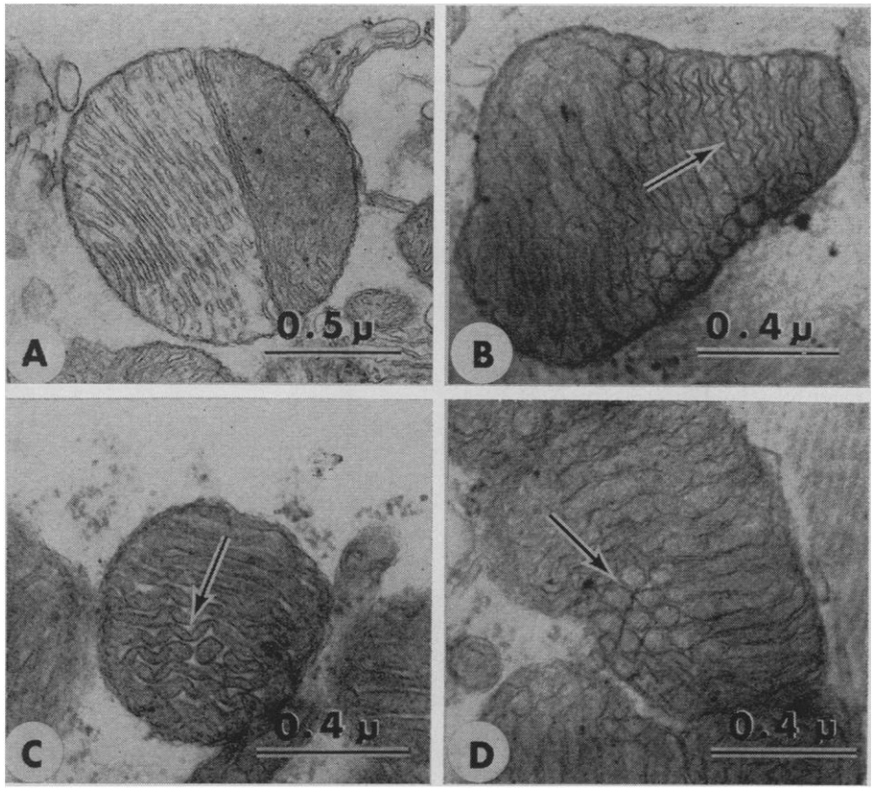

Fig. 4. The basic incubation medium was described in detail in the legend to Fig. 1. (A) A mitochondrion in a transitional state observed in the presence of antimycin (33 μg/ml) and sodium cyanide (7 mmole/liter); (B) mitochondrion with energized zigzag configuration of the cristal membrane observed in the presence of 10 m*M* sodium succinate; (C) mitochondrion with energized-twisted configuration of the membrane obtained in the presence of 10 m*M* sodium succinate; (D) mitochondrion with a paracrystalline array of the cristal membrane observed in the presence of 10 m*M* sodium succinate.

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3. The term configurational state, as used in the text, refers to the appearance in electron micrographs of the inner (cristal) membranes of the mitochondrion.
4. A nonenergized state of the mitochondrion prevails under nonenergizing conditions, for example, when electron transfer is blocked by anaerobiosis or when energy conservation is rendered impossible by an uncoupling reagent like dinitrophenol. The energized state of the mitochondrion can be induced and maintained by either electron transfer or adenosine triphosphate hydrolysis.
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Electron Spin Resonance Signals in Injured Nerve

Abstract. *Under certain conditions nerves (such as the frog sciatic) exhibit electron spin resonance signals with several unusual properties: (i) variable g value and linewidth, (ii) anisotropic g tensor, and (iii) g value dependence on temperature. Such a signal must be due to a small ferromagnetic crystal formed when the nerve is subjected to pressure, such as that due to mechanical injury.*

Although a number of normal animal tissues exhibit electron spin resonance (ESR) signals due to free radicals associated with enzymatic oxidation-reduction systems (*1*) in nerves, such signals are either absent or at least undetectable by means of present spectrometers. During investigations designed to determine whether ESR signals might arise in excised frog sciatic nerves during conduction of impulses, the absence of a normal ESR signal was confirmed under conditions of maximum available instrument sensitivity, both in the resting nerve and during conduction. However, in the course of these investigations, intense ESR signals at *g* values which varied between 2.05 and 2.30, with rather wide linewidths (100 to 400 gauss), appeared in several nerve preparations. Accordingly, a study was undertaken to determine the origin and properties of these signals. The results show that the signals arise in both frog sciatic nerve and lobster ventral nerve cord as a result of mechanical injury of the nerve, such as that due to pinching by means of forceps.

Most of the experiments were conducted with the excised sciatic nerve, dissected from cold-stored specimens of the bullfrog *Rana catesbeiana*. A few experiments were performed with the ventral nerve cord of the lobster *Homarus americanus*.

The excised nerves were examined in an ESR spectrometer operating at 9000 Mhz (modulation frequency, 100 khz), specially designed for maximum sensitivity in the presence of liquid water. The nerves were placed in either flat quartz cells (5 by 1 mm) or in a special cell consisting of a U-shaped glass tube which permitted initiation and detection of nerve impulses while the nerve was in place in the spectrometer cavity. Frog sciatic nerves were maintained in Ringer solution, and the lobster nerves were maintained in artificial seawater. The spectrometer was equipped with a temperature-regulating system which maintained the sample temperature to within ± 1°C of the indicated value. The spectrometer was operated in conjunction with a signal-averaging computer.

After the appearance of ESR signals during the handling of frog sciatic nerves, it became apparent that they frequently arose in association with mechanical injury, in particular, pressure such as that exerted by pinching with forceps. Examples of this effect are shown in Fig. 1, which illustrates that an intense ESR signal arises in the frog sciatic nerve after it has been pinched or stretched to the breaking point. By cutting off the injured portion we have observed that the signal is localized only in the injured portion of the nerve; the remainder of the nerve, like the original uninjured nerve, lacks an ESR signal.

Other treatment which elicits such signals in frog sciatic nerve is crushing (by means of pressure from a glass rod pressing against a flat glass surface), homogenization in a glass homogenizer, and splitting of the original nerve into two or more bundles (which inevitably involves stretching of the nerve). While studying 116 nerve preparations, we found that a signal could be elicited by these means in 80 samples.

One of the features of these signals is the considerable variability of shape and *g* value. In an effort to analyze

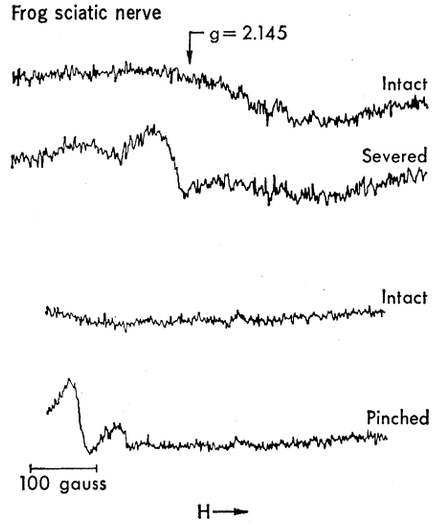

Fig. 1. Electron spin resonance signals from two different frog sciatic nerves (contained in a flat quartz ESR sample cell) before and after being severed by stretching (upper spectra) and before and after being pinched in forceps (lower spectra). Modulation amplitude: 16 gauss; temperature 15°C. Each of the signals represents a readout from a computer of average transients after summation by the computer of 50 successive spectra.

the source of this variability, we conducted a series of ESR determinations on single nerve preparations at different temperatures from −40° to +80°C. The signal is observed over this wide range of temperatures, but its *g* value is markedly dependent on temperature (Fig. 2). This figure shows that the position of the ESR signal shifts toward higher magnetic fields (lower values of *g*) as the temperature is increased (approximately linearly) and that the effect is completely reversible.

In order to study the origin of the ESR signal in the injured nerve, a number of nerves which exhibited the signal were sequentially cut in half (transversely, until very small); each time the two halves were studied for free radical content. If the nerve originally had a single ESR signal, the entire signal was always found in one half or the other—that is, the signal was never divided between the two nerve segments. By this means it has been possible, in some cases, to localize the ESR signal originally observed in the whole nerve in a segment as small as 0.3 mm on a side. When a small portion of an injured nerve which contains the ESR signal is isolated in this way, the signal intensity is essentially equal to that observed in the whole nerve. Hence the signal produced by the original injury is concentrated in a very