either myelination or blood-brain barrier (that is, more myelination or less barrier in spinal cord relative to brain in the newborn guinea pig).

The pattern of disease is significantly different in newborns of other species, where myelination of the CNS is less well developed at birth and in the neonatal period (*11*, *12*). In the neonatal monkey, the severe lesions of AE are found primarily in the hindbrain (paleoencephalon) and spinal cord (*13*), those regions which first become myelinated during embryonic development (*12*). This is in contrast to the high incidence of severe lesions in the cerebral hemispheres of adolescent and mature rhesus monkeys (*14*). The age-influenced susceptibility of a localized portion of the central nervous system to the induction of AE in the monkey is analogous to that described for the guinea pig (*11*, *12*). In the neonatal rat AE cannot be produced, and the CNS of neonatal rats appears devoid of encephalitogenic activity (*11*). These findings can also be correlated with a delay in myelination in the monkey and the rat, and thus provide further evidence for the concept of maturation of the CNS as a target organ being one of the necessary conditions for the induction of this disease. Whether a threshold amount of myelin alone, or whether maturity of the myelin already present as well, is required to provide the target organ is not known.

The role of maturity of the target organ has been considered above mainly in terms of degree of myelination; still to be examined are the maturation and general role of the blood-brain barrier in the mechanism of this disease. Thus, an age dependency might reflect differences in maturity of the route of sensitized cells, if not a humoral component, to the specific target organ.

Whatever mechanism causes the higher susceptibility of the paleoencephalon in neonatal animals, the greater success of transfer of AE with younger animals is not necessarily due to the radiosensitive barrier phenomenon (in mice) which apparently involves the receptivity of the environment of transplanted cells (*6*). The status (size and age) of the CNS as target organ may be equally important.

Thus, the failure to transfer AE in strain 2/N guinea pigs even when the adult donors are of an age and sex which regularly develop active disease (*8*), and when the recipients are as small as those strain 13/N guinea pigs which regularly show adoptive AE, would indicate that the young strain 2/N guinea pigs do not have a susceptible target in the CNS. This conclusion is especially tempting since such transfers of cells yield good adoptive tuberculin sensitivity in recipient strain 2/N animals. The test for tuberculin sensitivity determines that a given portion of transferred cells is indeed viable in the recipient. In addition, pools of cells are tested (*1*) for significant amounts of antigen by injection of portions from the pool into random-bred (Hartley strain) recipients. In that Hartley guinea pigs are as susceptible as, or more susceptible than, strain 13/N guinea pigs, they would show active AE if too much antigen were transferred with the cells. This controls for the possibility of active disease, since such histo-incompatible recipients cannot develop adoptive AE.

The foregoing experiments delineate the major variables now known to influence adoptive AE in guinea pigs; they furthermore serve to emphasize the simplicity and ease of reproducing the adoptive disease under the experimental conditions originally outlined (*1*).

The definition of the experimental variables operative in this autoimmune disease should be generally applicable in elucidating the role of cell-bound antibody in other diseases of immunologic etiology (*15*), and this procedure is suggested as a prototype for the induction of adoptive disease with other experimental models.

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4. Strains 13/N and 2/N refer to the Sewall Wright strains 13 and 2, currently raised at the NIH. Such designations, analogous to those used for inbred mice, become necessary as many centers of research are now breeding these animals. For the basic conditions for lymphoid transfers, see J. A. Bauer and S. H. Stone, *J. Immunol.* **86**, 177 (1961); N. E. Hyslop and S. H. Stone, *Fed. Proc.* **24**, 183 (1965).
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15. In contrast to the ease of transfer of AE, the difficulties encountered in attempts to transfer other autoimmune diseases (*3*) may be due to the chronicity and reversibility of a disease such as autoimmune thyroiditis contrasted to the acute and irreversible nature of AE.

1 December 1967

Mitochondrial Malate Dehydrogenase: Reversible Denaturation Studies

Abstract. *The malate dehydrogenase isoenzymes from the mitochondria of chicken hearts have been partially resolved into separate pools. Reversible denaturation, in concentrated guanidine hydrochloride, does not change the isoenzyme distribution in each pool. This result suggests that the electrophoretic differences among the isoenzymes are not solely conformational in origin.*

There is now considerable discussion of those enzymes whose multiple molecular forms are thought to be due to conformational variations of covalently identical proteins, and the term "conformers" has been suggested for these groups of isoenzymes (*1*). This conformer hypothesis has been applied to explain the existence of the multiple forms of chicken heart mitochondrial malate dehydrogenase (*1*), human placental and leukocyte mitochondrial malate dehydrogenase (*2*), *Neurospora* malate dehydrogenase (*3*), and chicken brain creatine kinase (*4*). Conformers may be considered to be special cases of the group of proteins that includes beef heart cytochrome *c* (*5*), human hemoglobin H (*6*), and mouse hemoglobins (*7*), for each of which there is evidence of two conformational states in equilibrium with each other.

We have recently suggested that reversible denaturation of nonequilibrating isoenzymes, after their partial or complete resolution, will provide information about the applicability of the conformer hypothesis (*8*). Our argu-

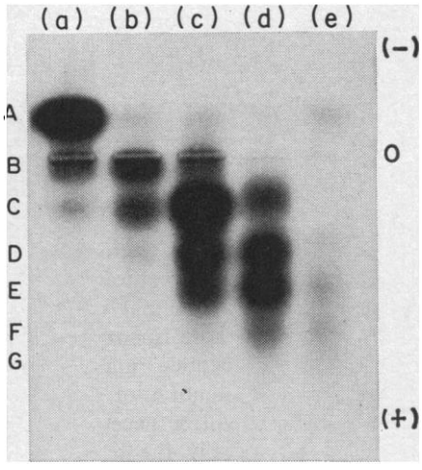

Fig. 1 (left). Photograph of starch-gel electrophoresis of mitochondrial malate dehydrogenase pools stained for enzyme activity. Samples from each of the five pools, (*a*) to (*e*), prepared from the shallow carboxymethyl-cellulose gradient described in the text were placed in the wells of a starch gel prepared as described in (*10*), except that 0.5 ml of 1*M* β-mercaptoethanol was added to the starch slurry during degassing. Electrophoresis was done for 19 hours, at 4°C, at 30 ma with tray buffers as described in (*10*). Staining with a sodium L-malate, diphosphopyridine nucleotide, phenazine methosulfate, and nitro blue tetrazolium solution was done as in (*10*). The bands of enzyme activity have been labeled *A* through *E*, going from the cathodal to the anodal direction, in correspondence with published work (*11*). Other bands (*F* and *G*) have been denoted by an extension of this nomenclature.

ment is based on the fact that separated conformers should be identical after complete unfolding in a denaturant and should, therefore, all renature to the same form or set of forms. The persistence of differences among pools of previously resolved isoenzymes, after reversible denaturation, implies the presence of differences which are not *solely* conformational. Conformational variations which are secondary to changes such as amino acid substitution, deamidation, and ligand heterogeneity would be expected to retain their identity under these conditions. The results reported here of studies on the denaturation and renaturation of chicken heart mitochondrial malate dehydrogenase (MDH) in guanidine hydrochloride suggest that the conformer hypothesis may not be an adequate explanation of the heterogeneity of this enzyme.

Mitochondrial MDH was partially purified from chicken hearts by aqueous extraction, ammonium sulfate fractionation, and diethylaminoethyl-cellulose and carboxymethyl-cellulose chromatography as described by Kitto and Kaplan (*9*). The first portion of the enzyme eluted from the carboxymethyl-cellulose column was concentrated and reapplied to a 2- by 75-cm carboxymethyl-cellulose column at *p*H 6.5. Elution from this column was effected with a linear gradient of potassium phosphate, ranging in concentration from 0.005 to 0.05*M*. Starch-gel electrophoresis at *p*H 7.0 with nitro blue tetrazolium staining for enzyme activity (*10*) was used for ascertaining the distribution of the mitochondrial MDH isoenzymes. On this basis the effluent from this shallow gradient was pooled into five fractions. We have labeled these (*a*) through (*e*) and their starch gel patterns are shown in Fig. 1. Fraction (*e*) corresponds to the front of the peak of eluted enzyme, and (*a*) to the back. Attempts at further resolution of these bands by repeated gradient elution and Sephadex chromatography gave some improvement, but only with a considerable loss of protein.

The five MDH isoenzymes previously designated *A*, *B*, *C*, *D*, and *E* (*11*) are shown in Fig. 1. In addition, two bands migrating more rapidly toward the anode, *F* and *G*, are seen clearly. In some runs with very heavily loaded gels, one or two bands even more anodal than *F* and *G*, but still slower than the supernatant MDH, were seen. Reversible denaturation studies have been done with the unfractionated mitochondrial MDH and with the five mitochondrial MDH pools.

The conditions studied by Chilson *et al.* (*12*) for maximizing the reversibility of denaturation of MDH by guanidine hydrochloride were modified in the following way: the enzyme was placed in 7.6*M* guanidine hydrochloride made up in 0.1*M* sodium citrate, *p*H 7.0, with 0.005*M* dithioerythritol, for 1 hour at room temperature. After several minutes of this exposure no MDH activity was detectable by the spectrophotometric assay (*10*) used throughout these studies. Renaturation was initiated by diluting the enzyme-guanidine solution 20-fold with 0.5*M* citrate, *p*H 7.0, containing 0.005*M* dithioerythritol. Kinetic studies, at room temperature, showed that the maximal recovery of activity was attained within 1 to 2 hours. Enzyme activity at 2 hours was therefore used as the index of the degree of renaturation effected.

Under these conditions, the percentage of enzyme renatured (as compared to control tubes in which the guanidine was added with the diluent) was 81 percent for pool (*a*), 82 percent for pool (*b*), 59 percent for pool (*c*), 49 percent for pool (*d*), and 29 percent for pool (*e*). These figures are based on the results of eight experiments. Since the percentage of renaturation was found to be inversely related to the enzyme concentration, an attempt was made to have approximately the same initial enzyme activities in all tubes. This condition was not obtained for

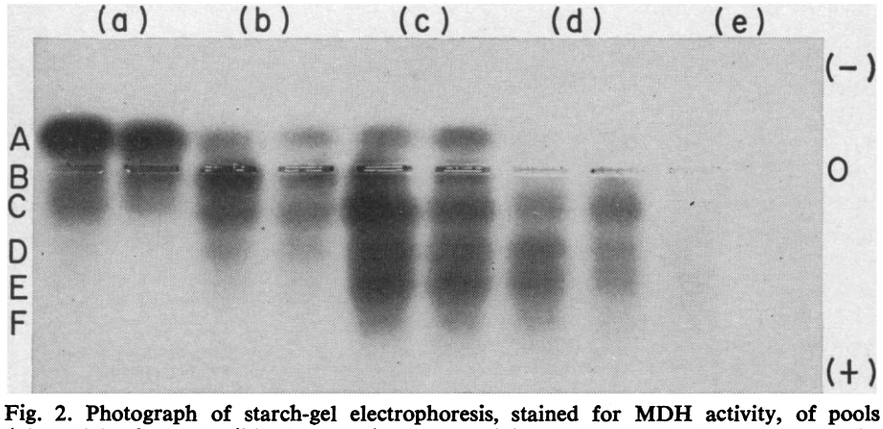

Fig. 2. Photograph of starch-gel electrophoresis, stained for MDH activity, of pools (*a*) to (*e*) after reversible denaturation by guanidine hydrochloride. For each pair the sample on the right is of enzyme that has been denatured and renatured. The sample on the left has not been exposed to concentrated denaturant. The activities of the (*e*) pools are faint in this photograph but were visible in the starch in the region of bands *D*, *E*, and *F*. The denaturation and renaturation process is as described in the text. Concentration of the samples 10- to 20-fold was done by vacuum dialysis, at 4°C, against the starch buffer before application to the gel. Electrophoresis and staining was done as described in the legend to Fig. 1, except that a current of 26 ma was applied for 18 hours.

pools (*b*) and (*e*) because of their relative diluteness. Thus, the recovery of activity in pools (*b*) and (*e*) has probably been somewhat enhanced relative to the other pools by this phenomenon.

The starch-gel electrophoretic patterns for each of these five pools before and after reversible denaturation with guanidine hydrochloride are shown in Fig. 2. The pattern of the isoenzymes has not been significantly altered by exposure to 7.6*M* guanidine hydrochloride. We have found this result in each of five experiments. Varying the experimental procedure, by using phosphate or glycylglycine buffer instead of citrate, and by exposing the enzyme to the 7.6*M* guanidine hydrochloride for periods as long as 6 days at 37°C, gave the same results. These experiments were done to lessen the possibility that anion stabilization (*13*) or kinetic effects (*14*) were interfering with the denaturation process. We have also placed enzyme from each pool in guanidine hydrochloride in small dialysis sacs and suspended these sacs for several hours in a common reservoir containing a small amount of the denaturant. Such cross dialyses do not change the banding pattern of each pool. Additionally, we have made various mixtures of the pools, with and without prior or subsequent exposure to the denaturing conditions, and studied the electrophoretic patterns of these mixtures. The resultant patterns were basically the sums of the component pools used.

These results suggest that the differences among the isoenzymes persist in concentrated guanidine hydrochloride, even after 6 days of exposure, and are difficult to reconcile with a primary conformational heterogeneity. The cross dialysis experiments tend to exclude an explanation in terms of ligand heterogeneity, unless such ligands were not equilibrated by dialysis.

In some reversible denaturation experiments we have seen slight changes in the *relative* intensities of the bands in each pool. The increased staining of band *A* in relation to bands *B* and *C* in the renatured pool (*c*) of Fig. 2, as compared to the relative intensities of the bands in the control pool, is an example of such a change. In pool (*d*), band *C* has been intensified with respect to bands *D* and *E* by the reversible denaturation process. As noted above, electrophoreses of various mixtures of pools, before and after denaturation, gave patterns which were the sums of component pools and were thus not suggestive of a rearrangement of nonidentical subunits (as with lactate dehydrogenase). Although such a rearrangement of subunits cannot be entirely excluded, we believe that these small changes in the relative intensities of bands are probably best explained by differential renaturability of the isoenzymes. The order of decreasing recoveries of enzyme activities, after denaturation, from pools (*a*) through (*e*) suggests a similar ordering in the renaturability of the bands *A* through *G*, which would account for these relative changes. In any case, this phenomenon does not change the main fact that the very major part of the separated isoenzymes renature to their original state, and not to identical sets of isoenzymes.

Using the conditions outlined by Chilson, Kitto, and Kaplan (*15*), we have studied the acid denaturation of the MDH isoenzymes. We have been successful in recovering 4 to 15 percent of the control enzyme activity of the unfractionated MDH after 1 hour of exposure to *p*H 2.0. However, renaturation has been successful for us for only the cathodal and neutral bands, not for the anodal ones, and without evidence of interconversions.

The interpretation of our acid denaturation studies is limited by the fact that we obtained lower yields than were obtained in those studies that suggested an acid-catalyzed conversion of anodal proteins to proteins migrating at cathodal or more neutral positions (*1*, *16*). However, it should be noted that enzyme stains of starch-gel electrophoreses give only semiquantitative data and must be cautiously interpreted, especially if differential recovery of the several isoenzymes is occurring. Differential recovery would also affect complement-fixation assays based on enzyme activity (*1*). Rigorous proof of an interconversion of bands necessitates the demonstration of an increase in the *absolute* quantities of one or more bands. The many known covalent alterations in proteins which are catalyzed by protons (*17*) also restrict the interpretation of acid denaturation studies. The work on electrophoretic and optical rotation changes caused by iodination and on measurement of mean residue rotation at 233 mμ of the separated MDH bands (*1*) is open to several interpretations and alone is insufficient to establish the conformer hypothesis.

Tanford and his colleagues have shown for a large group of proteins that 6*M* guanidine hydrochloride produces random coil behavior, as determined by viscosity, sedimentation, optical rotation, and other measurements (*18*). Proteins containing more than one subunit would be dissociated as well. Thus, unless chicken heart mitochondrial MDH is very different from those other proteins in its behavior in concentrated guanidine hydrochloride, the demonstrated stability of the patterns of the MDH pools to this denaturant must be considered in the eventual explanation of the origin of these isoenzymes. Further work on peptide mapping and structural determination of highly resolved enzymes will be necessary to establish unequivocally the basis of this molecular heterogeneity.

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19. We thank Dr. J. C. Kereztesy and D. Rogerson of this Institute for help in the large-scale extraction and separation steps. A.N.S. held a PHS Special Research Fellowship (1-F3-AM-35, 712-01) from NIAMD during part of this work.
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