Brain Enolases as Specific Markers of Neuronal and Glial Cells

Abstract. *There are three distinct enolase isoenzymes in brain: neuron-specific enolase (NSE), formerly referred to as neuron-specific protein, which is specifically localized in neurons, a nonneuronal enolase (NNE), and a third hybrid form. Light microscopy with immunocytochemical techniques has permitted localization of nonneuronal enolase. The NNE is located in glial cells with no staining of endothelial cells or neurons. Thus, NSE and NNE can be used as specific metabolic markers for neurons and glial cells, respectively.*

The discovery that a neuron-specific protein (NSP) was a neuron-specific form of the glycolytic enzyme enolase [2-phospho-D-glycerate hydrolase (E.C. 4.2.1.11)] (*1, 2*) has provided an added dimension to its role as a neuronal marker (*3*). It is therefore appropriate that the term NSP be amended. We shall refer from now on to this protein as neuron-specific enolase (NSE). Isolated and characterized from rat, cat, and human brain (*2, 4, 5*), NSE has been demonstrated by structural, immunological, and functional criteria to be identical to the 14-3-2 protein originally isolated by Moore (*6*). Structural analysis has demonstrated that NSE is a dimer composed of two apparently identical 39,000-dalton subunits.

When whole brain extracts are fractionated by DEAE-cellulose ion-exchange chromatography, the total enolase activity is resolved into three peaks (*2*). The most acidic fraction is NSE, and the least acidic is designated nonneuron specific enolase (NNE). Fraction NNE has been purified, characterized, and shown to be structurally and immunologically distinct from NSE (*7*). The NNE fraction is also a dimer but it is larger than NSE, being composed of two apparently identical 43,500-dalton subunits (*7*). Both NSE and NNE are therefore the products of separate genes with the respective subunit designations being $\gamma\gamma$ and $\alpha'\alpha'$. The DEAE enolase fraction of intermediate acidity is designated as the hybrid since it appears to be composed of one NNE subunit and one NSE subunit ($\alpha'\gamma$).

We report the localization of NNE in brain tissue by means of the peroxidase-antibody to peroxidase (PAP) technique (*8*). We found that only glial and epithelial elements contained NNE, an indication that, in mature rat brain, the enolase isoenzymes are morphologically separated from one another. NNE apparently is a characteristic glial enzyme and NSE is the neuronal equivalent. These two enolase enzyme forms can serve as specific biochemical markers for the respective cell types.

Antiserum to NNE was produced in New Zealand white rabbits in the manner described for the production of antiserum to NSE (*4*). The immunological reactivity of antiserum to NNE with each brain enolase enzyme form was assessed by immunoprecipitation (*9*) (Fig. 1). Antiserum to NNE does not react at all with NSE but does cross react to a significant extent with the hybrid enolase form. In an effort to obtain a serum more specifically directed against NNE the antiserum to NNE was incubated with the partially purified hybrid enolase to sequester the antibodies that react with this enolase form (*10*). Immunoprecipitation analysis of the hybrid-adsorbed antiserum to NNE (Fig. 1) revealed that its reactivity with the hybrid enolase was negligible, while it retained most of its reactivity with NNE. The hybrid-adsorbed antiserum to NNE was used exclusively in the immunocytochemical analyses. In an effort to demonstrate the distinction between NSE and NNE cellular localization, we also stained some sections with antiserum to NSE. This serum does not react at all with NNE, but does react well with the hybrid enolase (Fig. 1). The antiserum to NSE was not adsorbed with the hybrid enolase fraction.

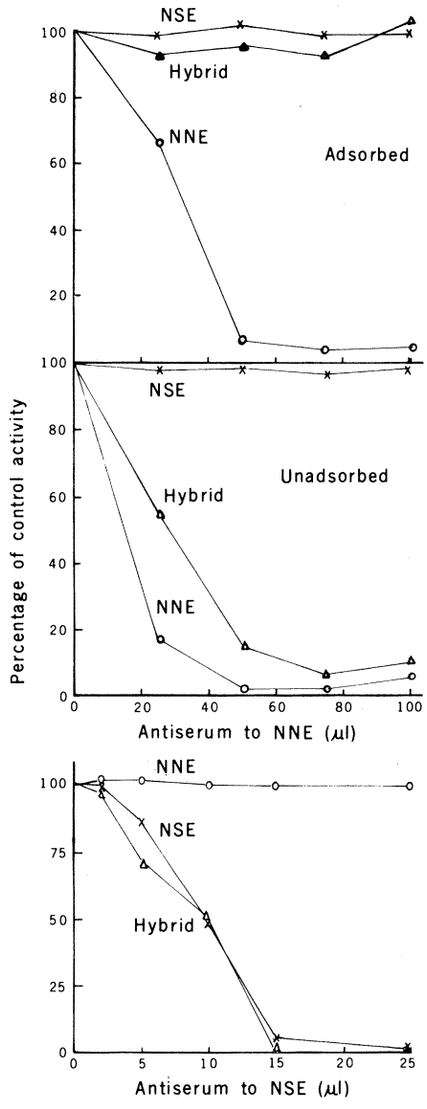

Fig. 1. Immunological reactivity of each rat brain enolase fraction with antiserum to NSE and antiserum to NNE. In each case 0.5 unit of each partially purified brain enolase fraction was incubated with the indicated amount of antiserum. The amount of enolase activity remaining in the supernatant was determined (*8*).

Rats were anesthetized with pentobarbital and perfused through the aorta with a solution of picric acid and formalin (*11*); the brain was dissected, and the sections were postfixed for 3 to 6 hours, and embedded in polyester wax (*12*). Sections (4 to 8 μm thick) were stained according to the unlabeled antibody enzyme method (*8*) with incubation procedures as described (*13*). Serum obtained prior to immunization of the experimental rabbits served as a control.

The stained sections revealed that adsorbed antiserum to NNE reacted only with nonneuronal elements, including astrocytes, ependymal cells, tanycytes, and choroid plexus epithelial cells. The cytoplasm stained throughout as should be expected for a soluble glycolytic enzyme. Some nuclear staining was encountered but the dense cytoplasmic reaction often obscured the nucleus. Fine detail was visible such as perivascular endings, pial endfeet, and the branching processes of astrocytes. Glial cells that stained for NNE embraced Purkinje cell bodies (Fig. 2C) and were identical in position and morphology to glial cells seen in Golgi impregnated tissue (*14*). Adjacent sections stained with NSE antiserum served to emphasize the strict delineation of neurons as compared to glia (Fig. 2). In no case did the adsorbed antiserum to NNE stain neurons; conversely, the antiserum to NSE stained only neurons. With fixation conditions used, no significant staining of oligodendroglia was observed. With both serums, the corpus callosum and white matter remained relatively stain-free. There was a moderate variation in staining intensity in any given cell class of neurons or glia.

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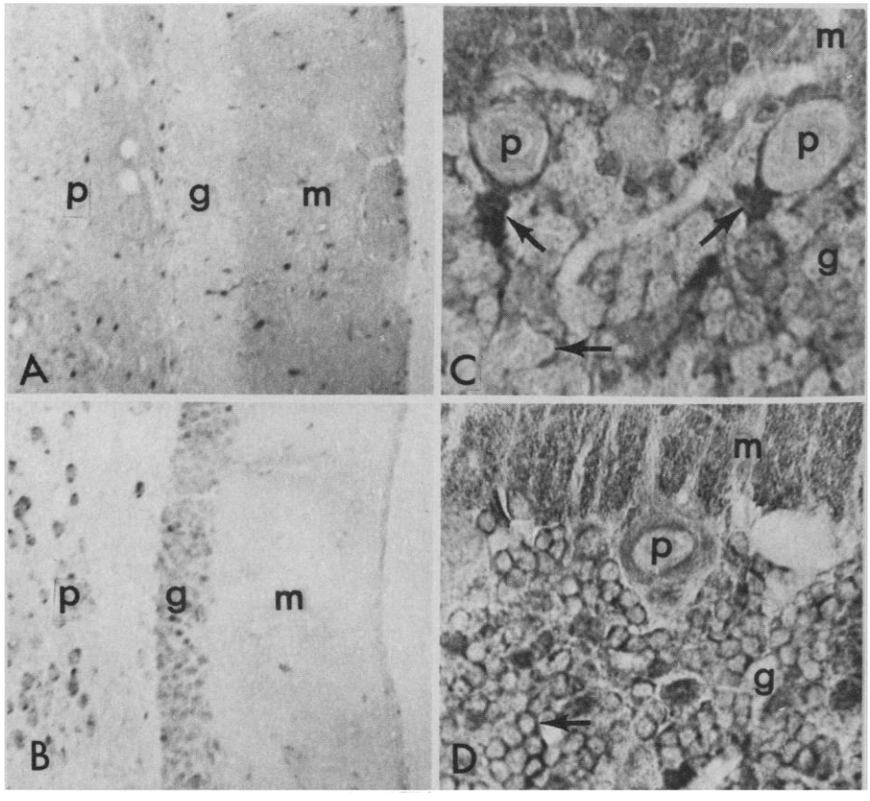

Fig. 2. Photomicrographs of sections of rat brain stained for NSE or NNE by the unlabeled antibody enzyme method and counterstained with cresyl violet (*m*, molecular layer; *g*, granule cell layer; *p*, polymorphic layer; *P*, Purkinje cell). (A) Hippocampus stained for NNE shows positive glial cells throughout all three layers (× 115). (B) Hippocampus stained for NSE shows positive neurons located primarily in *p* and *g* layers (× 115). (C) Cerebellum; antiserum to NNE reveals two glial cells (oblique arrows) whose processes envelop Purkinje cell bodies (*P*) (× 470). (D) Cerebellum; NSE-treated section (× 470). Both Purkinje cells and granule cells (horizontal arrows) are unstained in (C), but positively stained in the NSE-treated section (D).

Whether this reflects an actual variation in the enolase content of cells or loss of antigen during fixation is unknown. The presence of occasional nuclear staining, however, suggests some lability of the antigen during fixation. The results of our study clearly establish that the enolase isoenzymes of brain are strictly segregated, with NNE being present only in glia and NSE only in neurons.

NNE is immunologically very similar to liver enolase since liver extracts react well with antiserum to NNE (*7*), and antiserum to NNE stains liver sections quite efficiently. The question of whether NNE is identical to liver enolase must await a detailed structural comparison of the two enzymes. NNE is therefore probably not specific to the nervous system but within the nervous system it is confined to the glial elements.

At present, the localization and properties of the hybrid enolase form are not clear. Structural studies suggest that the hybrid may be an artifact of tissue disruption since it has been shown that it can be generated by mixing NSE and NNE. If the hybrid enolase is not an artifact of preparation then the fact that antiserum to NSE, which reacts well with hybrid (Fig. 1), stains only neurons indicates that this enzyme form is localized in neuronal elements.

A number of molecular markers have been used to characterize various cell types in nervous tissue by immunocytochemical methods (*14, 15*). Neurotransmitter related enzymes have been utilized as neuronal markers (*16*) but are only useful on specific subsets of neurons containing the respective enzyme. The enolase isoenzymes NSE and NNE provide the capability of using the same enzyme function to delineate neuronal and glial cells. The existence of generalized neuronal and glial markers may also be useful in tissue culture work where morphological criteria alone do not suffice for identification of cell types. Studies in mouse and chick brain show that NSE appears at a point in neuronal development coincident with functional maturation (*17*) indicating that the neuronal enolase function is a highly differentiated one.

Studies are now possible in which the neuroanal and glial enolase could be examined simultaneously during the course of brain development. Because of their importance as enzymes in a crucial metabolic pathway in brain, the brain enolases should prove useful as molecular markers, as well as providing perhaps basic answers to questions of cell lineage and differentiation in the nervous system.

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9. Antiserums to NNE and NSE were tested by immunoprecipitation in order to assess their reactivity with each brain enolase enzyme fraction. In each case a portion of the partially purified DEAE fraction of each respective enzyme form containing 0.5 unit of enzyme activity was incubated with the indicated amount of antiserum. The samples were incubated for 2 hours at room temperature and then centrifuged at 25,000*g* for 20 minutes. The resulting supernatant fraction was assayed for enolase activity. Activities were presented as a percentage of the activity obtained by incubating in the presence of 60 μl of serum from an unimmunized rabbit, which served as the control. Enolase activity was assayed by the direct spectrophotometer assay (*2*). A unit of enolase activity is defined as that amount of enzyme catalyzing the formation of 1 μmole of product per minute at 25°C.
10. The antiserum to NNE was adsorbed with the partially purified hybrid enolase fraction. Three units of hybrid enolase activity were added to each 1 ml of antiserum to NNE. This solution was incubated at 25°C for 2 hours and then held at 4°C overnight. The resulting suspension was centrifuged at 25,000*g* for 30 minutes, and the supernatant was collected. This process was repeated until no further precipitate formed (usually two precipitations). The resulting serum was designated hybrid-adsorbed antiserum to NNE. Antiserum to NNE that was not adsorbed did occasionally yield a very faint neuronal staining, again indicating that the hybrid enolase form may be present in neurons. Adsorption of antiserum to NSE with the hybrid was not attempted since it was expected that the resulting serum would be sharply reduced in its NSE titer.
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Ingestion of Crude Oil: Sublethal Effects in Herring Gull Chicks

Abstract. *A single small oral dose of Kuwait or South Louisiana crude oil caused cessation of growth, osmoregulatory impairment, and hypertrophy of hepatic, adrenal, and nasal gland tissue in herring gull chicks living in a simulated marine environment. These findings suggest that ingesting crude oil causes multiple sublethal effects that might impair a bird's ability to survive at sea.*

The total annual influx of oil to the oceans of the world was estimated to be between 5 and 10 million metric tons in the early 1970's and was considered to be increasing as a result of increased transportation and expanded exploitation of regions in which drilling might be more hazardous (*1*). Although spectacular oil spills account for only a small fraction of the total, the number of oiled sea and shore birds that wash up after a spill indicates that oil is acutely toxic. Furthermore, the low survival rate of apparently healthy birds that have been cleaned of oil and released suggests delayed toxic effects (*2*). These delayed effects could be caused by oil that is swallowed or aspirated when birds preen or ingest contaminated food and seawater (SW). Crocker *et al.* (*3*) have reported that the ingestion of crude oil inhibits salt and water transport in the intestine of saline-adapted ducklings. Although they did not measure plasma electrolytes, we have found that crude oil does impair plasma osmoregulation in SW-stressed (nonadapted) ducklings (*4*). These data suggest one possible mechanism of toxicity in a species that is not strictly marine. Laboratory experiments with true sea birds present many problems. Although most species of gulls are coastal, they can osmoregulate in a simulated marine environment (Fig. 1); they thus provide a convenient laboratory model for studying the toxicity of oil to sea birds. We now report that a single small dose of crude oil inhibits growth and impairs plasma osmoregulation in immature herring gulls (*Larus argentatus*). Crude oil also causes hypertrophy of adrenal, hepatic, and nasal gland tissue and induction of hepatic microsomal cytochrome P-450 activity.

Herring gull chicks about 3 to 4 weeks of age were captured on Old Man Island, off Cutler, Maine, and transported to the Mount Desert Island Biological Laboratory. Birds were housed in sheds and fed whole unsalted herring and 50 percent SW, which were freely available. After 5 days, birds were divided into two experimental groups and one control group of six birds each. Birds in the experimental groups were given by intubation a single oral, 0.2-ml dose (dose equivalent to 0.3 ml per kilogram of body weight) of either a Kuwait crude (KC) oil containing 22 percent aromatics or a heavier South Louisiana crude (SLC) oil containing 17 percent aromatics (*5*). This dose appears to be environmentally realistic and is well below that which causes lipid pneumonia in waterfowl (*6*). Experimental and control birds were then moved to separate sheds, and all were given 100 percent SW (440 meq per liter of Na^+, 9.4 meq per liter of K^+, and 940 mosmoles per kilogram of water to drink. Each day, gulls were weighed and 0.2-ml blood samples (wing vein) were taken. One day after being dosed, a control and an SLC gull died; 4 days later another SLC gull died after exhibiting

Table 1. Effect of crude oil (a single oral 0.2-ml dose) on herring gull intestinal transport, organ weights, and activity of Na^+,K^+-adenosine triphosphatase (Na^+,K^+-ATPase). Each value is given as the mean ± standard error of the mean; statistical comparisons are made between experimental and control groups and tested according to unpaired *t*-tests.

Item	Group		
	Control (N = 5)	KC (N = 6)	SLC (N = 4)
Intestinal transport†			
0.1 m*M* cycloleucine			
3 minutes	163 ± 19	178 ± 14	115 ± 9*
10 minutes	337 ± 34	395 ± 66	243 ± 17*
0.1 m*M* cycloleucine + 10 m*M* leucine			
3 minutes	89 ± 4	83 ± 8	83 ± 3
Inhibition (%)	43 ± 5	48 ± 7	27 ± 4*
1 m*M* glucose			
3 minutes	1865 ± 232	2181 ± 320	1199 ± 315
1 m*M* glucose + 0.1 m*M* phlorizin			
3 minutes	883 ± 114	915 ± 56	771 ± 28
Inhibition (%)	49 ± 9	53 ± 8	29 ± 14
Intestine			
Weight/surface area‡	4.48 ± 0.14	4.23 ± 0.17	4.14 ± 0.33
Na^+,K^+-ATPase activity (specific)			
Weight§	2.82 ± 0.40	2.32 ± 0.62	1.76 ± 0.61
Area‖	2.50 ± 0.30	2.22 ± 0.56	1.64 ± 0.94
Nasal salt gland (one)			
Gland weight			
Weight (mg)	387 ± 22	436 ± 28	451 ± 32
Weight/body weight (mg/kg)	454 ± 17	563 ± 14**	575 ± 37*
Na^+,K^+-ATPase activity			
Weight (specific)§	47 ± 2	36 ± 1**	32 ± 2**
Gland (total)¶	1037 ± 74	908 ± 76	817 ± 50*
Liver			
Organ weight			
Weight (g)	22.3 ± 1.4	29.6 ± 2.8*	32.6 ± 2.0**
Weight/body weight (g/kg)	0.26 ± 0.01	0.38 ± 0.02**	0.42 ± 0.01**
Cytochrome P-450 activity#	420 ± 80	780 ± 120*	910 ± 130*
Adrenal glands (two)			
Gland weight			
Weight (mg)	83 ± 4	95 ± 13	103 ± 6*
Weight/body weight (mg/kg)	0.98 ± 0.08	1.27 ± 0.17	1.33 ± 0.11*

†Nanomoles per square millimeter of tissue. ‡Milligrams per square millimeter. §Micromoles of inorganic phosphate (P_i) per milligram of protein per hour. ‖Micromoles of P_i per square millimeter per hour. ¶Micromoles of P_i per gland. #Nanomoles per milligram of protein. *$P < .05$. **$P < .01$.

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