High-Resolution Proton Nuclear Magnetic Resonance Studies of the Exchangeable Resonances of the *lac* Repressor Headpiece†

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ABSTRACT: The exchangeable hydrogens of the N-terminal 51 amino acid headpiece (HP) of the *lac* repressor have been examined with high-resolution ^1H NMR. Resonances from 35 ± 3 protons are observable in ^2H_2O solutions at p^{2}H 4.2. The exchange rates for some of these have been measured over the p^{2}H range 1.5–5. In H_2O, resonances from 70 ± 6 protons are observable at pH 3.2 and 35 ± 5 protons at pH 7.1. The temperature dependence of the spectrum at near neutral pH shows the existence of a group of ~22 protons particularly slow to exchange. The pH dependence of the spectrum in H_2O solution over the range 1–11 is discussed. Results are compared with NMR studies of the nonexchanging protons and CD and IR results.

Since Geisler & Weber (1977) showed that it was possible to selectively cleave the *lac* repressor at amino acid 51 or 59 and that the smaller fragment is able to bind deoxyribonucleic acid (DNA), there has been considerable interest in determining the structure of this fragment and the nature of its interaction with DNA [for a discussion of studies to date, see Ribeiro et al. (1981b)]. NMR studies of exchangeable hydrogens are of particular interest for studies of secondary and tertiary structure, since these hydrogens are commonly found in the hydrogen bonds which stabilize such structures.

Experimental Procedures

The 51 amino acid headpiece was prepared from *lac* repressor isolated from *Escherichia coli* as previously described (Geisler & Weber, 1977; Ribeiro et al., 1981a). Samples in H_2O were prepared by dialysis of the HP against a large volume of buffer and then adjustment of the pH with appropriate amounts of NaOH or HCl solution. For ^2H_2O solutions, the HP was lyophilized from H_2O buffer near neutral pH and then dissolved in ^2H_2O at the appropriate pH immediately before the measurement was to be made. Approximately 5 min was required to prepare the sample and shim the field before beginning the first accumulation. All solutions were 0.25–0.50 mM HP and had a total ionic strength of ~1 M, primarily KCl with high phosphate (300 mM) or low phosphate (5–15 mM), as noted. Ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) were added at 5×10^{-6} M and cacodylic acid at 2×10^{-4} M (for H_2O solutions only) as preservatives. H_2O samples had ~10% ^2H_2O added for field-frequency locking. The pH of samples in H_2O was read before and after NMR measurements were made. The p^{2}H of samples in ^2H_2O was read after the exchange measurements were finished and is reported as such, uncorrected for isotope effects. All measurements unless otherwise noted were made at room temperature, 23 ± 2 °C, and samples in ^2H_2O were kept at 23 ± 2 °C between measurements.

NMR measurements were made on the modified Bruker HXS-360 spectrometer with Nicolet 1180 data system at the Stanford Magnetic Resonance Laboratory. Spectra in ^2H_2O were taken with a selective preirradiation of the residual solvent peak for 2 s, with a total delay between pulses of 3.64 s. Spectra in H_2O were taken with the Redfield 21412 sequence (Redfield et al., 1975), and for some samples further solvent suppression was obtained by audiofrequency notch filtering (Marshall et al., 1979). Temperature of samples during measurements was controlled with a Bruker BST 100 control unit with estimated accuracy ±2 °C and stability ±0.5 °C.

IR measurements were made on a compressed pellet of the HP lyophilized from H_2O with ~1 M KCl. Measurements were taken on the Nicolet 7199 FTIR instrument at the University of California, San Diego. CD measurements were made on 0.06 mM solutions in high-phosphate H_2O and ^2H_2O buffers.

Results and Discussion

The exchange rates for labile hydrogens in amino acids with solvent water are very pH dependent. However, the minimum rate for any group exposed to solvent is thought to be about 10^{-1} min^{-1} (Wüthrich & Wagner, 1979). These rates are sufficiently fast that virtually no signals would be seen in ^2H_2O solutions, even if freshly prepared. However, hydrogens which are protected from contact with solvent through position in the interior of a protein or through participation in hydrogen bonding may have much lower exchange rates. Figure 1 shows the downfield part of the spectrum of the HP freshly dissolved in ^2H_2O at p^{2}H 4.2, representing the first 0.5 h after addition of solvent, and the spectrum in H_2O at pH 3.2. Integration of the ^2H_2O spectrum yields an estimate of 35 ± 3 exchangeable hydrogens, while the H_2O spectrum has 70 ± 6 exchangeable hydrogens. At the pH of the H_2O measurements, it is possible that many solvent-accessible resonances are seen; however, those resonances observed in ^2H_2O solution must come from residues protected from solvent. For most small proteins, the majority of amino acid side chains containing labile hydrogens are on the surface and freely exposed to solvent. If this is true for the HP, then the majority of the 35 protons observed must come from backbone amides, indicating about two-thirds of the HP backbone is involved in secondary structure. Since OH protons are expected to exchange too rapidly to be observed at any pH, the 70 protons observed in H_2O represent a majority of the 84 possible NH protons in the HP, both backbone and side chain.

The exchange rates of labile hydrogens are also related to the stability of the macromolecular superstructure. In the extremely stable β-sheet region of bovine pancreatic trypsin inhibitor (BPTI), exchange times of many months have been observed (Karplus et al., 1973). Approximate exchange rates

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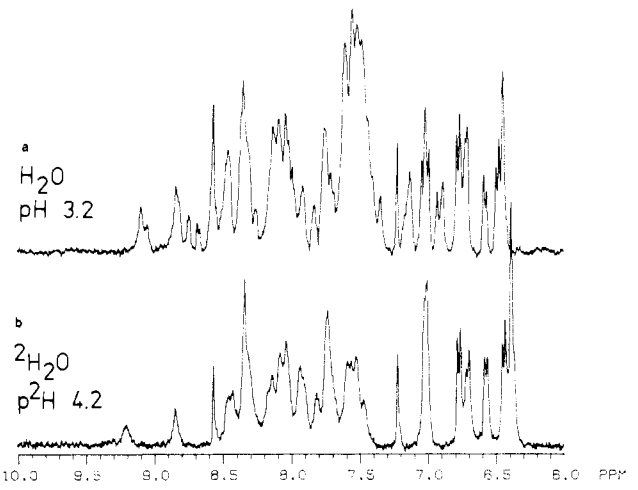

FIGURE 1: ^{1}H NMR spectra of 0.25 mM HP in (a) H_2O solution (pH 3.2, low-phosphate buffer, 23 °C), using the Redfield method and audiofrequency notch filtering, and (b) freshly dissolved ^2H_2O solution (p^{2}H 4.2, low-phosphate buffer, 23 °C). Both spectra were taken with a 3.64-s delay between pulses and are the average of 2000 scans. The carrier frequency was at ~7.8 ppm. Peak area measurements were referenced to the histidine peak at 7.22 ppm area = one and to the tyrosine-7 and -47 peaks at 7.0 ppm area = four.

Table I: Rates of Exchange of Some Amide Resonances in the HP

	rates (min^{-1} × 10^3)[a]				
pH	1[b]	2	3	4	5
1.6	0.57	1.8, 0.46	1.8, 0.45	0.61	0.79
2.6	0.19	0.38	1.8, 0.27	0.30	1.5, 0.55
3.6	0.42	0.49	0.45	0.38, 0.22	1.8, 0.21
4.5	0.66	1.2	1.5	1.2	3.3, 0.98

[a] Lines 2–5 correspond to more than one proton, and where nonexponential decays were observed they were approximated by two exponentials, both reported. [b] Resonance positions at p^{2}H 1.6 are (1) 8.77, (2) 8.40, (3) 8.30, (4) 7.72, and (5) 7.54 ppm.

for peaks observed in ^2H_2O solution as a function of p^{2}H are shown in Table I. Although the data are primarily qualitative due to overlap of many of the peaks, the basic dependence upon p^{2}H is clear.

The detailed interpretation of exchange rates of amide protons is complex and controversial. Two basic models have been proposed: the "breathing protein" model, proposing that exchange occurs through formation of "open" structures in which exchange occurs rapidly with solvent and then closes to the native form again, and the "solvent penetration" model in which solvent water molecules diffuse into the protein interior, to be exchanged with hydrogen-bonded protons through local structural fluctuations. It has been shown that the pH dependence is not sufficient to distinguish these models when a proper multistate formulation is used (Wagner & Wüthrich, 1980a). The exchange rates for the HP are higher than have been observed for other small proteins (Karplus et al., 1973; Richarz et al., 1979; Campbell et al., 1975; Stellwagen & Shulman, 1973; Patel & Canuel, 1976) and suggest that the structure is relatively flexible. This is consistent with previous observations that sharp lines are observed for the HP bound to intact repressor (Wade-Jardetzky et al., 1979; Buck et al., 1978) and that its unfolding with temperature occurs in a continuous fashion (Wemmer et al., 1981).

Although exchange rates in HP solutions at p^{2}H >6 are too fast to allow their measurement in ^2H_2O, spectra may still be obtained in H_2O solutions. In this case, the requirement for observation, slow exchange with solvent, reduces to $k_{exg} \lesssim 100$ s^{-1}. Figure 2 shows the spectrum of the HP in H_2O at pH 7.1. The number of labile protons observed has been reduced to 35 ± 5, approximately the same number which were observed in ^2H_2O at lower pH. A further classification of exchange rate is possible through observation of saturation transfer from solvent H_2O. Only resonances for which spins are exchanged with water several times (rates >1 s^{-1} but <100 s^{-1}) during presaturation will be strongly affected. Figure 2 also shows the spectrum obtained with 4-s presaturation of H_2O and the difference between this and the normal spectrum. There are 13–15 resonances affected, and hence in fairly rapid exchange with solvent, with ~20 in slow exchange. Examination of the temperature dependence of the spectrum provides a further approach for identification of more stable regions of a macromolecule. Figure 3 presents spectra of the HP in H_2O solution at pH 7.5 for several temperatures. Many changes are observed in the 20–40 °C temperature range, reflecting the loss of approximately 15 resonances through the increasing rate of exchange with solvent. In the temperature range 40–60 °C, the remaining resonances, representing ~22 protons, show only slight broadening. This set is probably involved in hydrogen bonds of a particularly stable part of the HP. It is interesting to note that only small shifts are noted in the resonances of *non*exchangeable residues over the 20–40 °C range (Wemmer et al., 1981), implying that the structure giving rise to the unusual tyrosine and methyl shifts is stable over this range and is probably the same as gives rise to the "stable" group of amide resonances in H_2O. The temperature-induced unfolding in the HP shows an isotope effect, a decrease, in the unfolding temperature of ~10 °C in H_2O vs. ^2H_2O, as monitored by chemical shifts of Tyr-17 and circular dichroism. This is reasonable for a structure which must be maintained through hydrogen bonds.

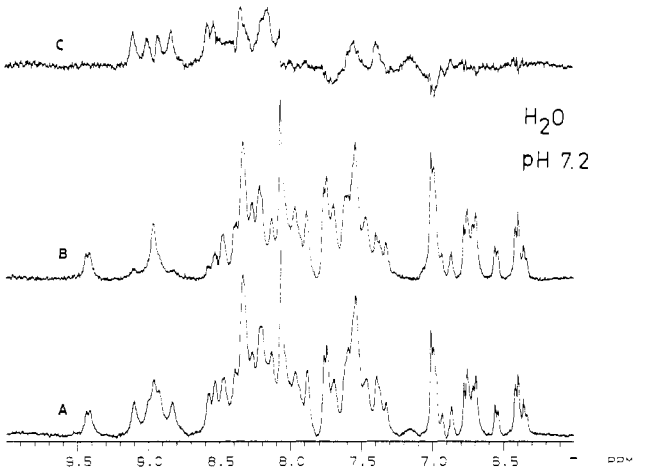

FIGURE 2: ^{1}H NMR spectra of 0.26 mM HP in H_2O (high-phosphate buffer, pH 7.2, 23 °C) (A) without presaturation of H_2O solvent and (B) with 4-s selective preirradiation of H_2O); (C) difference between (A) and (B). Both spectra were taken with the Redfield method with audiofrequency notch filtering with a 5.64-s delay between pulses, 6000 scans. The carrier frequency was at ~9.75 ppm. Negative peaks at 7.0 and 7.75 ppm in the difference spectrum are probably from NOE's generated by irradiation of α protons under the water line.

As mentioned above, there is a gradual decrease in the number of protons observable in H_2O solution with increasing pH. This is consistent with the increasing exchange rates measured at low pH in ^2H_2O solutions. At pH 9.5, the number observable is reduced to ~20 protons, with a chemical-shift distribution similar to that at pH 7.5 and 45 °C. It seems likely that this again represents the set of most stable hydrogen bonds in the HP. At higher PH, the number is further reduced until at pH ~11 no amide resonances are observed. Chemical

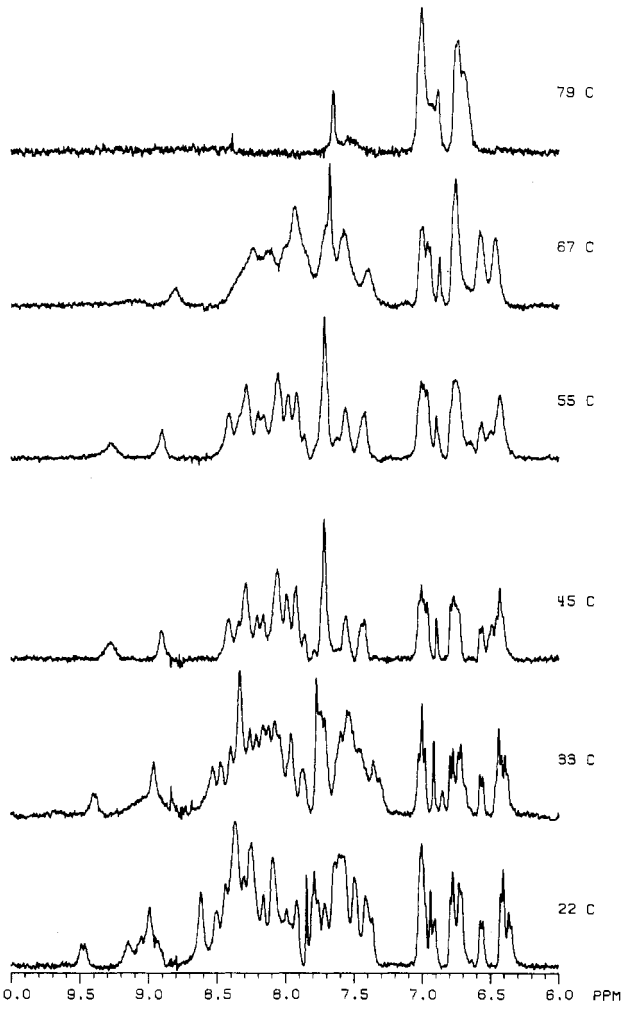

FIGURE 3: ^1H NMR spectra of 0.2 mM HP in H_2O (high-phosphate buffer, pH 7.5) as a function of temperature. All spectra were taken with the Redfield technique, 2.82-s delay between pulses, 2000 scans. The carrier frequency was in the 8.7-ppm region.

shifts of nonexchangeable resonances show that unfolding is occurring beginning at pH ~10 (Ribeiro et al., 1981b).

The temperature dependence of unfolding observed through the nonexchangeable resonances is not significantly different at pH 3, 7.8, and 9.5. This suggests that the structure and its stability are not significantly affected by pH below 10.

The pH dependences of chemical shifts of the most downfield-shifted amides are shown in Figure 4. Unfortunately due to the very crowded nature of the 7–8.4-ppm region, it has not been possible to follow titrations in this region. The resonances observable in ^2H_2O solution show only weak pH dependence over the observable pH range. The shifts of nonexchangeable resonances have also been observed and are reported in detail elsewhere (Ribeiro et al., 1981b). The shifting of the resonance occurring at 8.8 ppm at pH 1 has a pK_a of 4.8 and perhaps represents a side-chain carboxyl, hydrogen bonded to an amide, either side chain or backbone (Bundi & Wüthrich, 1979). It has approximately the same pK_a as the group responsible for the shifts observed of Met-42 SCH_3. This resonance disappears slowly with increasing temperature or pH and is observed in ^2H_2O solutions. The lines between 8.6 and 8.7 ppm shift with an apparent $pK_a \sim 2.1$, approximately what is expected for titration of the C terminus. However, the shift is in the opposite direction of that expected for the amide of this residue (Wüthrich & Wagner, 1979). It is possible that the shifts arise from through-space effects. The further shifts near pH 6 have

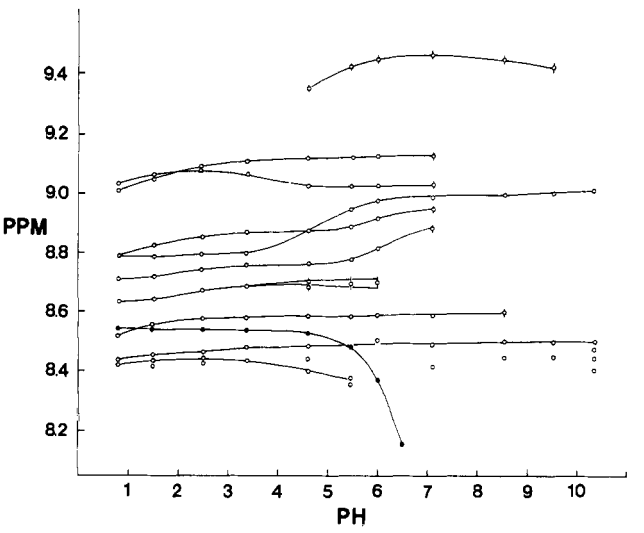

FIGURE 4: pH dependence of the resonance positions of the downfield-shifted protons in the HP in H_2O (high-phosphate buffer, 23 °C). Note that many resonances observable at low pH are not observable at higher pHs. Several resonances shift into or out of the 8.2–9.4-ppm region which cannot be followed over the entire pH range. The solid circles correspond to the C-2 porton of histidine-29, $pK_a = 6.6$.

apparent pK_a values of 6–7 and might be related to titration of the histidine, $pK_a = 6.6$. Several other lines shift in this pH range (resonances at 9.0, 8.8, and 8.5 ppm), but the curves are not characteristic of titrations. These are at approximately the correct pH to arise from changes in the overall charge of the protein as one or more of the six carboxylic acids become protonated. The resonance at 8.55 ppm comes from the histidine-29 C-2 proton and shows a pK_a of 6.6, consistent with the value measured in ^2H_2O solution (Ribeiro et al., 1981b).

From the spectrum in Figure 1b, it is possible to measure coupling constants for some of the downfield resonances. Most of those observable fall in the 3–6-Hz range, too small for β-sheet structures, but consistent with helical secondary structures. In addition, protons in β-sheet structures tend to be rather strongly downfield shifted and slow to exchange (Wüthrich & Wagner, 1979). In the HP, there are only two stable resonances between 8.5 and 10.5 ppm, where many β-sheet proton resonances in BPTI occur, making the existence of extensive classical β structures in the HP unlikely. Further evidence for helical structure in the, HP comes from CD and IR spectra. The CD spectrum has been discussed previously (Geisler & Weber, 1977), showing a strong positive band at 190 nm and negative bands at 205 and 220 nm, typical of helical structures. The infrared spectrum (as KCl pellet) shows amide bands at 1653 and 1535 cm^{-1}, again typical for helical structures.

From the preceding discussion of the particular features of the exchangeable protons in the HP, several definite conclusions can be drawn. From the number of slowly exchanging protons in ^2H_2O, we know that there is extensive secondary and perhaps some tertiary structure, involving approximately two-thirds of the 51 amino acids. The temperature dependence shows that a set of resonances exists which are more temperature and pH stable than others observed, suggesting that the structure has two distinct regions, which have different stability and unfold at different temperatures. A significant temperature shift in unfolding upon deuteration is observed, consistent with a structure arising primarily from hydrogen bonding. As assignments are made for these amide protons, it should be possible to provide significantly more detail of the structure.

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References

Buck, F., Rüterjans, K., & Beyreuther, K. (1978) *FEBS Lett. 96*, 335-338.
Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 299-311.
Campbell, I. D., Dobson, C. M., & Williams, R. J. P. (1975) *Proc. R. Soc. London, Ser. B, 189*, 485-502.
Geisler, N., & Weber, K. (1977) *Biochemistry 16*, 938-943.
Karplus, S., Snyder, G. H., & Sykes, B. D. (1973) *Biochemistry 12*, 1323-1329.
Marshall, A. G., Marcus, T., & Sallos, J. (1979) *J. Magn. Reson. 35*, 227-230.
Patel, D. J., & Canuel, L. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1398-1402.
Redfield, A. G., Kunz, S. D., & Ralph, E. K. (1975) *J. Magn. Reson. 19*, 114-117.
Ribeiro, A. A., Wemmer, D., Bray, R. P., Wade-Jardetzky, N. G., & Jardetzky, O. (1981a) *Biochemistry 20*, 818-823.
Ribeiro, A. A., Wemmer, D., Bray, R. P., & Jardetzky, O. (1981b) *Biochemistry* (preceding paper in this issue).
Richarz, R., Sehr, P., Wagner, G., & Wüthrich, K. (1979) *J. Mol. Biol. 130*, 19-30.
Stellwagen, E., & Shulman, R. G. (1973) *J. Mol. Biol. 75*, 683-695.
Wade-Jardetzky, N., Bray, R. P., Conover, W. W., Jardetzky, O., Geisler, N., & Weber, K. (1979) *J. Mol. Biol. 128*, 259-265.
Wagner, G., & Wüthrich, K. (1979a) *J. Mol. Biol. 130*, 31-37.
Wagner, G., & Wüthrich, K. (1979b) *J. Mol. Biol. 134*, 75-94.
Wemmer, D., Ribeiro, A. A., Bray, R. P., Wade-Jardetzky, N. G., & Jardetzky, O. (1981) *Biochemistry 20*, 829-833.
Wüthrich, K., & Wagner, G. (1979) *J. Mol. Biol. 130*, 1-18.

Efflux of γ-Aminobutyric Acid by Synaptic Plasma Membrane Vesicles Isolated from Rat Brain†

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ABSTRACT: Synaptic plasma membrane vesicles isolated from rat brain were "actively" loaded with γ-aminobutyric acid (GABA) by a process driven by a sodium ion as well as a chloride ion gradient (both out > in). Subsequently, dilution-induced efflux from these vesicles was monitored. This efflux was 2-3-fold enhanced by the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone and 4-6-fold by externally added GABA. The ability of GABA to stimulate was most pronounced when both sodium and chloride ions were present in the dilution medium. The dependency of efflux of γ-aminobutyric acid on both internal sodium and chloride ions was demonstrated by three independent types of experiments: (a) preloading of the vesicles with sodium and chloride ions markedly stimulated γ-aminobutyric acid efflux. (b) Conditions presumably enhancing the internal sodium concentration, such as dilution in sodium-containing media in the presence of nigericin, enhanced the efflux about 10-fold. Such stimulation was not observed with vesicles previously loaded with sodium. Efflux into chloride-containing media was only slightly enhanced by triphenyltin chloride; on the other hand, this compound strongly inhibited efflux into chloride-free media. (c) A freeze–thaw technique was used to load GABA passively into the vesicles (thus without the need to introduce the external sodium and chloride required for the active loading). The efflux from such vesicles was dependent on the simultaneous presence of internal sodium and chloride ions. It is concluded that the efflux of γ-aminobutyric acid is in many aspects symmetrical with its influx [Kanner, B. I. (1978) *Biochemistry 17*, 1207-1211]. It appears that in order for γ-aminobutyric acid to interact with the carrier both sodium and chloride have to be present on the same side as the γ-aminobutyric acid. The simplest way to account for these and the previous data is to postulate cotransport of sodium, chloride, and γ-aminobutyric acid through the carrier.

Membrane vesicles isolated from various bacterial and mammalian cells have proved extremely useful for the study of active transport (cf. Kaback, 1974; Aronson & Sacktor, 1974; Hopfer et al., 1973; Colombini & Johnstone, 1974; Lever, 1977; Rudnick, 1977). Some of their advantages include the possibility of using well-defined energy sources and the lack of metabolism and storage in subcellular organelles. Recently, the use of membrane vesicles has been extended to those apparently originating from the synaptic plasma membrane (Kanner, 1980) for the study of sodium-dependent neurotransmitter transport in rat brain (Kanner, 1978; Kanner & Sharon, 1978). These transport systems have been implicated in the termination of transmitter action on postsynaptic receptors (Iversen, 1971). These latter transport studies have provided direct evidence that the general concept that solute accumulation can be achieved by cotransport with ions (Crane, 1965; Riggs et al., 1958; Mitchell, 1963) also applies to brain. Thus, it appears that the electrochemical potential gradient of Na^+ serves as a direct driving force for the transport of GABA[1] (Kanner, 1978) and L-glutamic acid

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