be said that microtubules apparently play an important role in determining the mobility and topography of receptor sites on cell membranes (Yahara and Edelman, 1975; Nicholson, 1976).

Refer ›nces

Adler, A. J., Greenfield, N. J., and Fasman, G. D. (1973), *Methods Enzymol. 27,* 675.
Attwood, D., Florence, A. T., and Gillan, J. M. N. (1974), *J. Pharm. Sci. 63,* 988.
Cann, J. R., and Hinman, N. D. (1975), *Mol. Pharmacol. 11,* 256.
Cann, J. R., and Hinman, N. D. (1976), *Biochemistry 15,* 4614.
Chen, Y.-H., Yang, J. T., and Martinez, H. M. (1972), *Biochemistry 11,* 4120.
Eipper, B. A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2283.
Eipper, B. A. (1974), *J. Biol. Chem. 249,* 1407.
Florence, A. T., and Parfitt, R. T. (1971), *J. Phys. Chem. 75,* 3554.
Frigon, R. P., and Lee, J. D. (1972), *Arch. Biochem. Biophys. 153,* 587.
Frigon, R. P., and Timasheff, S. N. (1975), *Biochemistry 14,* 4567.
Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8,* 4108.
Hinman, N. D., and Cann, J. R. (1976), *Mol. Pharmacol. 12,* 769.
Hinman, N. D., Morgan, J. L., Seeds, N. W., and Cann, J. R. (1973), *Biochem. Biophys. Res. Commun. 52,* 752.
Lee, J. C., Corfman, D., Frigon, R. P., and Timasheff, S. N. (1975), *Ann. N.Y. Acad. Sci. 253,* 284.
Lee, J. C., Corfman, D., Frigon, R. P., and Timasheff, S. N. (1978), *Arch. Biochem. Biophys. 185,* 4.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Ludeña, R. F., and Woodward, D. O. (1975), *Ann. N.Y. Acad. Sci. 253,* 272.
Nicholson, G. L. (1976), *Biochim. Biophys. Acta 457,* 57.
Seeman, J. L., Tedesco, J. L., Lee, T., Chan-Wong, M., Muller, P., Bowles, J., Whitaker, P. M., McManus, C., Tittler, M., Weinreich, P., Friend, W. C., and Brown, G. M. (1978), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37,* 130.
Ventilla, M., Cantor, C. R., and Shelanski, M. (1972), *Biochemistry 11,* 1554.
Weisenberg, R. C., Borisy, G. G., and Taylor, E. (1968), *Biochemistry 7,* 4466.
Wilson, L., and Meza, I. (1972), *J. Cell Biol. 55,* 285a.
Yahara, I., and Edelman, G. M. (1975), *Ann. N.Y. Acad. Sci. 253,* 455.

Platelet 5-Hydroxytryptamine Transport, an Electroneutral Mechanism Coupled to Potassium†

Gary Rudnick* and Pamlea J. Nelson

ABSTRACT: Transport of 5-hydroxytryptamine into plasma membrane vesicles isolated from porcine blood platelets is stimulated when a potassium gradient (in > out) is imposed across the vesicle membrane. This stimulation occurs in the absence of measurable electrical potential across the membrane. Addition of valinomycin induces a membrane potential of approximately 50 mV (interior negative) as estimated by uptake of the lipophilic cation triphenylmethylphosphonium, but has surprisingly little effect on 5-hydroxytryptamine transport. Addition of 2,4-dinitrophenol dissipates the valinomycin-induced membrane potential. In the absence of valinomycin, 2,4-dinitrophenol has no effect on 5-hydroxytryptamine transport but valinomycin and 2,4-dinitrophenol together inhibit transport, probably by dissipation of the K^+ gradient. These results are consistent with an electroneutral mechanism in which 5-hydroxytryptamine influx is directly coupled to potassium ion efflux and argue against an electrogenic mechanism in which there is a net influx of positive charge with 5-hydroxytryptamine.

Transport of 5-hydroxytryptamine (5-HT)[1] is an energy-dependent, carrier-mediated process with an absolute requirement for Na^+ and Cl^- in the external medium (Sneddon, 1969; Lingjaerde, 1971; Rudnick, 1977). Similarities between 5-HT transport into platelets and synaptosomes and brain slices have led to the proposal that the same transport system is present in both tissues (Sneddon, 1973; Paasonen, 1968). A previous report from this laboratory demonstrated that plasma membrane vesicles isolated from human blood platelets accumulate 5-HT to concentrations approximately 100 times greater than in the external medium (Rudnick, 1977). Accumulation is stimulated by imposition of a potassium ion gradient (in > out) across the vesicle membrane. This observation can be interpreted in one of two ways: (1) K^+ efflux stimulates transport by creating an electrical potential (interior negative) across the membrane, or (2) K^+ interacts directly with the 5-HT carrier, which couples K^+ efflux to 5-HT uptake. The

† From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510. *Recieved April 11, 1978; revised manuscript received July 18, 1978.* This work was supported by a Grant-in-Aid from the American Heart Association and with funds contributed in part by the Hartford, Connecticut Chapter.

[1] Abbreviations used: 5-HT, 5-hydroxytryptamine; $TPMP^+$, triphenylmethylphosphonium.

0006-2960/78/0417-4739$01.00/0

direct coupling of solute transport to K^+ gradients has been proposed for transport of sugars by small intestine and transport of amino acids by mouse ascites and yeast cells (Crane, 1965; Eddy, 1968; Eddy et al., 1970) but the role of K^+ in these systems is now believed to be indirect. Previous observations in this laboratory (Rudnick, 1977) led to the suggestion that 5-HT transport in platelets is electrogenic (charge crosses the membrane with 5-HT) and the stimulatory effect of K^+ was via generation of a membrane potential (negative inside). In the present report we show that under these conditions no significant membrane potential was formed. This result, along with others presented in this communication, suggest that 5-HT is transported by an electroneutral mechanism in which K^+ interacts with the transporter directly.

Materials and Methods

Preparation of Membrane Vesicles. Fresh porcine blood was obtained at a local slaughterhouse and collected into 175 mL of acid citrate–dextrose solution (Aster & Jandl, 1964) per L of blood. Platelet rich plasma was prepared by repeated centrifugation at 200*g* for 15 min at room temperature. Membrane vesicles were then prepared by the procedure of Barber & Jamieson (1970) with the following exception. Lysis of glycerol-loaded platelets was performed at 37 °C in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 5 mM $MgSO_4$, and 0.0033% deoxyribonuclease I. This modification and the use of fresh blood consistently yield preparations of membrane vesicles capable of accumulating 5-HT to apparent concentrations 300 to 500 times that of the external medium. Vesicles were suspended in 10 mM Tris-HCl (pH 7.5), containing 0.25 M sucrose at a concentration of 2–4 mg of membrane protein per mL, frozen in liquid N_2, and stored at 80 °C.

Transport Assays. 5-HT transport was measured at 25 °C as described previously (Rudnick, 1977). Triphenylmethylphosphonium ($TPMP^+$) uptake was determined by the same technique using 20 μM [^{3}H]TPMP Br (38 000 cpm/nmol) in the assay mixture. Cellulose–acetate filters (Millipore EHWP) were used to measure $TPMP^+$ uptake. Apparent concentration gradients of substrates taken up by the vesicles were calculated on the basis of 12.2 μL of internal volume per mg of membrane protein (Rudnick, 1977).

[1,2-^{3}H]-5-HT was obtained from New England Nuclear. Deoxyribonuclease I, 2,4-dinitrophenol, and valinomycin were obtained from Sigma Chemical Co. [^{3}H]Triphenylmethylphosphonium bromide was kindly donated by Dr. H. R. Kaback and the Isotope Synthesis group of Hoffmann-La Roche, Inc.

Results

To determine the possible connection between membrane potential and concentration gradients of potassium ion imposed across the vesicle membrane, membrane potential was monitored by measuring the distribution of the lipophilic cation triphenylmethylphosphonium ($TPMP^+$). Lipophilic ions were first used for this purpose by Grinius et al. (1970) and are believed to rapidly distribute across lipid bilayers and biological membranes in response to an electrical potential difference across the bilayer. $TPMP^+$ has been used to measure potentials across bacterial and mammalian membranes which are inaccessible to direct measurement with microelectrodes (Schuldiner & Kaback, 1975; Bakker et al., 1976; Grollman et al., 1977; Lever, 1977a). In these systems, $TPMP^+$ is accumulated in the vesicle in response to an electrical potential (interior negative) imposed across the membrane.

When platelet plasma membrane vesicles equilibrated with potassium phosphate buffer are diluted into NaCl medium, $TPMP^+$ reaches a concentration in the vesicle interior approximately equal to its external concentration, demonstrating that a K^+ gradient across the vesicle membrane is not sufficient for the formation of a membrane potential (Figure 1). This observation suggests that the membrane is not significantly more permeable to K^+ and Cl^- than to Na^+ and phosphate. Similar results (data not shown) are obtained when vesicles are diluted into NaSCN medium, which was previously shown to stimulate 5-HT accumulation (Rudnick, 1977), indicating that the stimulation was probably not due to formation of a membrane potential (interior negative). In contrast, addition of the K^+-specific ionophore valinomycin to the vesicles, which would be expected to catalyze electrogenic efflux of K^+, leads to accumulation of $TPMP^+$ to an apparent concentration over six times higher than that of the external medium (Figure 1). Assuming that the distribution of $TPMP^+$ reflects the membrane potential, the magnitude of that potential can be calculated from the Nernst equation, and, for the experiment shown in Figure 1, the valinomycin-induced potential is approximately 50 mV (interior negative). The observation that the potential estimated from $TPMP^+$ uptake is less than that predicted from the imposed K^+ gradient (78 mV) is most easily explained by the movement of other ions, such as Na^+ or phosphate, in addition to K^+. The proton conducting ionophore 2,4-dinitrophenol would be expected to collapse the valinomycin-induced potential by allowing protons to enter the vesicles. This is observed in Figure 1, where 2,4-dinitrophenol prevents valinomycin-induced $TPMP^+$ accumulation.

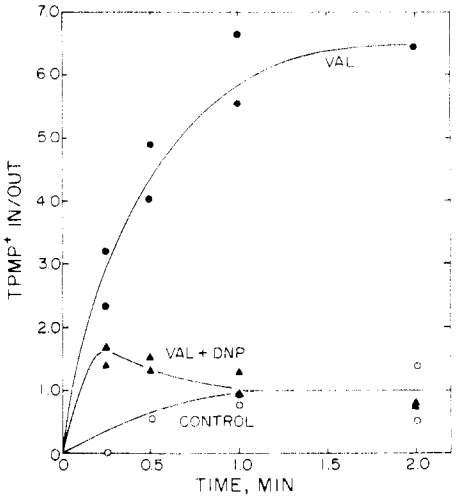

FIGURE 1: Uptake of $TPMP^+$ by platelet membrane vesicles. Membrane vesicles equilibrated with 0.1 M potassium phosphate buffer, pH 6.7, containing 1 mM $MgSO_4$ were suspended to a concentration of 3.4 mg of membrane protein/mL and diluted into a 20-fold excess of 0.1 M NaCl containing 1 mM $MgSO_4$ and 20 μM [^{3}H]TPMP Br (38 000 cpm/nmol). At given times, vesicles were diluted, filtered, washed, and counted as described previously (Rudnick, 1977) using cellulose acetate filters. (○) Control; (●) vesicles contained 12 nmol of valinomycin/mg of membrane protein; (▲) vesicles containing valinomycin were diluted into assay solution containing 1 mM 2,4-dinitrophenol.

Although valinomycin causes a large increase in membrane potential under these conditions, it has negligible effects on 5-HT transport. As shown in Figure 2, valinomycin causes only a 12% increase in the maximum level of 5-HT accumulated inside membrane vesicles. If 5-HT transport were electrogenic, as previously proposed (Rudnick, 1977), valinomycin would be expected to increase accumulation of 5-HT six- to sevenfold. This experiment also demonstrates the increased activity of vesicles prepared from fresh platelets, when compared with

previous results (Rudnick, 1977) using vesicles prepared from outdated platelets. Addition of 2,4-dinitrophenol in the absence of valinomycin has no effect on 5-HT transport, demonstrating that transport is not dependent on any previously existing membrane potential (unless that potential is formed by a proton diffusion gradient). When both valinomycin and 2,4-dinitrophenol are added, however, transport is markedly inhibited (Figure 2). Under these conditions both K^+ and H^+ would be expected to flow down their electrochemical gradients, with the result that net exchange of internal K^+ for external H^+ occurs while the Na^+ gradient is maintained. 5-HT accumulation in the presence of valinomycin plus 2,4-dinitrophenol in the experiment shown in Figure 2 was roughly equal to the 35- to 50-fold concentration gradient of 5-HT accumulated in response to a NaCl gradient (out > in) when $K^+_{in} = K^+_{out}$. Accumulation of 5-HT, therefore, seems to be coupled much more closely to the K^+ concentration gradient than to the membrane potential.

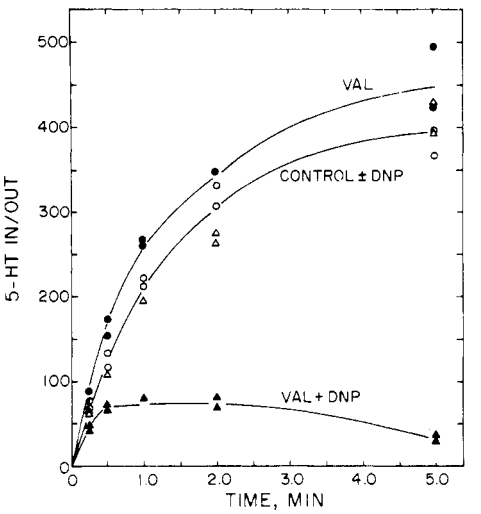

FIGURE 2: Effects of valinomycin and 2,4-dinitrophenol on 5-HT transport. Transport assays were performed as Figure 1, except that 0.13 μM [1,2-^{3}H]-5-HT (15 300 cpm/pmol) replaced [^{3}H]TPMP Br and reaction mixtures were filtered on nitrocellulose filters. (○) Control; (●) vesicles contained 12 nmol of valinomycin/mg of membrane protein; (△) assay mixture contained 1 mM 2,4-dinitrophenol; (▲) vesicles containing valinomycin were diluted into assay mixture containing 2,4-dinitrophenol.

Discussion

The response of platelet 5-HT transport to imposed gradients of K^+ is distinctly different from the behavior observed in other vesicle systems. In membrane vesicles prepared from *Escherichia coli* (Schuldiner & Kaback, 1975; Hirata et al., 1974), renal (Beck & Sacktor, 1975), and intestinal (Murer & Hopfer, 1974) brush border and cultured mouse fibroblast cells (Lever, 1977b), potassium gradients have little effect on transport in the absence of valinomycin. In these systems, a K^+ gradient (in > out) in the presence of valinomycin stimulates transport, an observation which has been used to demonstrate that these transport systems are electrogenic. In the case of platelet 5-HT transport, however, the stimulatory effect of a K^+ gradient (in > out) is independent of valinomycin, although generation of a membrane potential by a K^+ gradient requires valinomycin. Since K^+ does not exert its stimulatory effect via a membrane potential, a direct interaction of K^+ with the 5-HT transporter is indicated. Such an interaction was proposed by Sneddon (1969) on the basis of studies with intact platelets.

A possible alternative explanation for the lack of stimulation of 5-HT accumulation by a valinomycin-induced membrane potential (interior negative) is that increased Na^+ influx due to the induced potential dissipates the transmembrane Na^+ gradient. With an electrogenic transport system, the effects of simultaneously increasing potential and decreasing the Na^+ gradient could offset one another so that no stimulation would be observed with valinomycin. This alternative scheme does not, however, explain stimulation of 5-HT transport by a K^+ gradient in the absence of a membrane potential, an effect observed with all preparations of vesicles tested.

A direct role for K^+ has been proposed by Eddy & coworkers (Eddy, 1968; Eddy et al., 1970) for transport of glycine into mouse ascites-tumor cells and the yeast *Saccharomyces carlsbergensis* where in both cases efflux of K^+ accompanies glycine uptake. It is now believed that in these systems K^+ exits by an independent pathway to compensate for the positive charge which enters the cell with glycine, and that K^+ exit is not directly coupled to glycine transport (Morville et al., 1973; Seaston et al., 1976). Crane has proposed that internal K^+ drives sugar uptake in intestine by competing for the Na^+ site on the sugar transporter (Crane, 1965, 1977) but this suggestion is contradicted by observations in membrane vesicles isolated from intestinal brush border where K^+ gradients (in > out) have no effect on transport in the absence of valinomycin (Murer & Hopfer, 1974).

In contrast with other known Na^+ dependent transport systems, platelet 5-HT transport appears to be independent of membrane potential, suggesting that no net charge movement accompanies 5-HT transport across the membrane. Previous results suggest that Na^+ is cotransported into the cell or vesicle with 5-HT, which exists mainly as a cation at physiological pH (Rudnick, 1977). If 5-HT is transported by an electroneutral mechanism, and the protonated form of 5-HT is transported, these two positive charges must be balanced by the 5-HT transporter directly. It is known that Cl^- is required for 5-HT transport into intact platelets and membrane vesicles (Lingjaerde, 1971; Rudnick, 1977) and preliminary experiments in this laboratory indicate that Cl^- is effective only when present on the vesicle exterior.[2] Taken together with the effect of K^+ gradients, these results suggest that for each molecule of 5-HT transported, one Na^+ and one Cl^- are cotransported and one K^+ is counter-transported by the 5-HT transporter. If such a mechanism is correct, it raises two questions. First, is there a K^+ site on the transporter which is distinct from the Na^+ site, or do Na^+ and K^+ bind to the same site, but at different steps in the catalytic cycle? Second, since 5-HT transport occurs in the absence of K^+ (Rudnick, 1977), can another ion such as H^+ take the place of K^+ or does transport become electrogenic in the absence of K^+? These questions are currently under study.

The mechanism proposed here for platelet 5-HT transport may also apply to the high-affinity re-uptake of amine neurotransmitters into nerve endings. Neurotransmitter re-uptake systems have been studied mostly in synaptosomes and tissue slices. These experimental systems do not readily lend themselves to examination of the molecular aspects of transport. Moreover, the well known permeability of nerve membranes to K^+ (Baker et al., 1962; Blaustein & Goldring, 1975) will obscure any distinction between changes in membrane potential and K^+ gradients. Thus, previous conclusions that neurotransmitter re-uptake is electrogenic, or that K^+ affects transport only via the membrane potential (Martin, 1973; Blaustein & King, 1976) may need to be reevaluated in light of the current findings.

[2] Rudnick, G., unpublished observations.

References

Aster, R. H., & Jandl, J. H. (1964) *J. Clin. Invest. 43,* 843.
Baker, P. F., Hodgekin, A. L., & Shaw, T. I. (1962) *J. Physiol. 164,* 355.
Bakker, E. P., Rottenberg, H., & Caplan, S. R. (1976) *Biochim. Biophys. Acta 440,* 557.
Barber, A. J., & Jamieson, G. A. (1970) *J. Biol. Chem. 245,* 6357.
Beck, J. C., & Sacktor, B. (1975) *J. Biol. Chem. 250,* 8674.
Blaustein, M. P., & Goldring, J. M. (1975) *J. Physiol. 247,* 589.
Blaustein, M. P., & King, A. C. (1976) *J. Membr. Biol. 30,* 153.
Crane, R. K. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24,* 1000.
Crane, R. K. (1977) *Rev. Physiol. Biochem. Pharmacol. 78,* 99.
Eddy, A. A. (1968) *Biochem. J. 108,* 195.
Eddy, A. A., Indge, K. J., Backen, K., & Nowacki, J. A. (1970) *Biochem. J. 120,* 845.
Grinius, L. L., Jasaitis, A. A., Kadziauskas, Yu. P., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, L. M., & Vladimirova, M. A. (1970) *Biochim. Biophys. Acta 216,* 1.
Grollman, E. F., Lee, G., Ambesi-Impiombato, F. S., Meldolesi, M. F., Aloj, S. M., Coon, H. G., Kaback, H. R., & Kohn, L. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2352.
Hirata, H., Altendorf, K., & Harold, F. M. (1974) *J. Biol. Chem. 249,* 2939.
Lever, J. E. (1977a) *Biochemistry 16,* 4328.
Lever, J. E. (1977b) *J. Biol. Chem. 252,* 1990.
Lingjaerde, O., Jr. (1971) *Acta Physiol. Scand. 81,* 75.
Martin, D. L. (1973) *J. Neurochem. 21,* 345.
Morville, M., Reid, M., & Eddy, A. A. (1973) *Biochem. J. 134,* 11.
Murer, H., & Hopfer, U. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 484.
Paasonen, M. K. (1968) *Ann. Med. Exp. Biol. Fenn. 46,* 416.
Rudnick, G. (1977) *J. Biol. Chem. 252,* 2170.
Schuldiner, S., & Kaback, H. R. (1975) *Biochemistry 14,* 5451.
Seaston, A., Carr, G., & Eddy, A. A. (1976) *Biochem. J. 154,* 669.
Sneddon, J. M. (1969) *Br. J. Pharmacol. 37,* 680.
Sneddon, J. M. (1973) *Prog. Neurobiol. 1,* 151.

Proton Correlation Nuclear Magnetic Resonance Study of Anaerobic Metabolism of *Escherichia coli*†

T. Ogino,* Y. Arata,* S. Fujiwara, H. Shoun, and T. Beppu

ABSTRACT: Proton correlation nuclear magnetic resonance has been used to investigate anaerobic metabolism of glucose in *Escherichia coli* cells. The time course of the concentrations of six metabolites (ethanol, lactate, acetate, pyruvate, succinate, and formate) has been followed at the very early stage of fermentation, and used to discuss dynamical aspects of the mixed-acid fermentation of glucose by *E. coli*.

In a recent communication (Ogino et al., 1978) we have briefly reported that proton correlation NMR can be used to follow the time course of reactions in cellular systems in a nondestructive way from the very early stage of fermentation. ^{13}C and ^{31}P NMR have been shown to be quite useful in investigating the metabolism in living cells, cellular organelles, and intact tissues (Eakin et al., 1972; Moon & Richards, 1973; Kainosho et al., 1977; Dwek et al., 1977). ^{1}H nuclear magnetic resonance (NMR) has a great advantage that the sensitivity is much better than ^{13}C and ^{31}P NMR, and that ^{1}H nuclei exist virtually in all biologically important molecules. In a series of interesting experiments reported by Williams and co-workers (Daniels et al., 1974, 1976; Dwek et al., 1977), ^{1}H NMR spectra of a variety of compounds in dissected organs such as rat adrenal cortex were observed in the pulse Fourier transform (FT) mode. However, in order to detect a smaller quantity of compounds in biological systems where a large amount of water always exist, a dynamic range much wider than that attained in the pulse FT mode is required. ^{1}H correlation NMR has been recognized as one of most promising techniques for this purpose (Dadok & Sprecher, 1974; Gupta et al., 1974; Arata et al., 1976, 1978). In the present paper, we will discuss, on the basis of ^{1}H correlation NMR data, dynamical aspects of the mixed-acid fermentation of glucose by *E. coli*.

Materials and Methods

E. coli K12 was grown in M9 medium with glucose (0.1 M) as sole carbon source, or in nutrient broth. Cells were harvested at the exponential phase at a cell density of about 0.4×10^9/mL, washed once with the M9 buffer when nutrient broth is used, and resuspended at a density of 0.5 to 2.5×10^9/mL in the same buffer. About 0.5 mL of the suspension was placed in a standard 5-mm NMR tube, bubbled with N_2 gas, and the resultant inhomogeneous cell suspension in the tube was incubated anaerobically in an NMR spectrometer at a probe temperature of 30 °C. ^{1}H NMR spectra were measured with a JEOL PS-100 spectrometer operating at 100 MHz in the

† From the Department of Chemistry, University of Tokyo, Hongo, Tokyo, Japan (T.O., Y.A., and S.F.), and the Department of Agricultural Chemistry, University of Tokyo, Hongo, Tokyo, Japan (H.S. and T.B.). *Received June 20, 1978.*

0006-2960/78/0417-4742$01.00/0