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Storage of Dopamine and Acetylcholine in Granules of PC12, a Clonal Pheochromocytoma Cell Line†

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ABSTRACT: PC12, a clonal line of rat pheochromocytoma, stores acetylcholine and catecholamines in different granules and secretes each. These properties allow us to compare the mechanism of transmitter storage in two different types of storage granules produced by the same secretory cell. The mechanism of storage of dopamine in PC12 granules is similar to that for catecholamines in storage granules from adrenal medulla and mammalian brain. Transport of catecholamines into these granules is driven by a transmembrane pH gradient (inside acidic) and/or membrane potential, which are established by the activity of a proton-translocating Mg^{2+}-ATPase present in the membrane of the granules. Transport of dopamine into isolated PC12 granules was stimulated by ATP and inhibited either by proton ionophores that dissipate transmembrane pH gradients or by *N,N'*-dicyclohexylcarbodiimide, which inhibited an associated Mg^{2+}-ATPase activity. The proton ionophores caused a marked efflux of dopamine from the granules but reduced granular stores of acetylcholine to a much lesser extent. Treatment of intact cells with the ionophore nigericin caused an efflux from the granules of previously accumulated acetylcholine; however, nigericin did not induce an efflux of acetylcholine from isolated granules. We suggest that the acetylcholine-storing granules may have to interact with some particular cellular component to be susceptible to nigericin. PC12 cells took up dopamine, tyrosine, and choline and quickly converted the choline to acetylcholine and the tyrosine to dopamine. The newly accumulated or synthesized dopamine entered storage granules more readily than did newly synthesized acetylcholine.

PC12 is a clonal line of rat pheochromocytoma (Greene & Tischler, 1976). PC12 cells synthesize acetylcholine and catecholamines, store each in different granules, and exhibit a depolarization-induced secretion of each (Greene & Rein, 1977a,b; Schubert & Klier, 1977). These properties make it possible to compare the storage of transmitters in two different types of storage granules produced by the same secretory cell. In this paper we report the use of PC12 cells for such studies.

Earlier studies with cell-free preparations of chromaffin granules and synaptic vesicles indicate that the transport of catecholamines into these particles is driven by a transmembrane pH gradient (inside acidic) and/or membrane potential, which are established by the activity of a proton-translocating Mg^{2+}-ATPase present in the membrane of the catecholamine-containing storage granules (Bashford et al., 1976; Johnson & Scarpa, 1976a, 1979; Casey et al., 1977; Flatmark & Ingebretsen, 1977; Toll & Howard, 1978). Here we present evidence that this mechanism of catecholamine transport and storage in granules also operates in intact cells; we also describe some ways in which the granular storage of acetylcholine

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differs from that of catecholamines.

Materials and Methods

Chemicals. ATP, eserine sulfate, iodoacetic acid, oligomycin, ouabain, pargyline, reserpine, and valinomycin were purchased from Sigma Chemical Co., and DCCD[1] was from Eastman Kodak. [^{3}H]Choline chloride (84 Ci/mmol) and [^{3}H]dopamine (33 Ci/mmol) were obtained from New England Nuclear; *S*-adenosyl[1-^{3}H]methionine (15 Ci/mmol) and [1-^{3}H]tyrosine (40 Ci/mmol) were obtained from Amersham. The following were obtained as gifts: FCCP from Du Pont, nigericin from Hoffmann-La Roche, S-13 from Monsanto Chemical Co., and desipramine from Pharmaceutical Corp.

Cell Culture. The clonal rat pheochromocytoma line, PC12, was obtained from Dr. D. Schubert. The cells were grown at 37 °C under an atmosphere of 11% CO_2 and 89% air in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 5% horse serum, 50 μg/mL streptomycin, and 50 units/mL penicillin on plastic culture dishes or flasks. The cells were used for the various experiments 4 to 5 days after subculturing, just prior to becoming confluent.

Electron Microscopy. The cells were suspended in a cacodylate-buffered fixative (Graham & Karnovsky, 1966) overnight at 4 °C, washed several times in 0.1 M sodium cacodylate, pH 7.2, and treated at room temperature for 30 min with 1% osmium tetraoxide (Stevens Metallurgical Co.) in 0.1 M sodium cacodylate, pH 7.2. After stepwise dehydration with ethanol and propylene oxide, the cells were embedded in Araldite 502. Samples were sectioned on an LKB Ultratome III ultramicrotome and sections stained with uranyl acetate and lead citrate. A Hitachi HU-11A electron microscope was used.

Isolation of Storage Granules. The cells were washed with 5 mL of ice-cold 0.32 M sucrose and 10 mM sodium phosphate, pH 7.4, per plate and harvested in the same buffer. A cell homogenate containing 1–10 mg of protein per mL was obtained by 15 up and down passes of a tight-fitting pestle in a Dounce homogenizer. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The supernatant suspension was centrifuged at 20000*g* for 30 min. The resulting pellet (P_2), which contained the storage granules, was kept on ice until use.

Incubation of Cells in Defined Buffers. The cells were incubated at 37 °C while still attached to the plastic dishes or flasks in a cell incubation buffer consisting of 60 mM sucrose, 10 mM glucose, 130 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 25 mM Hepes, pH 7.3. Where indicated, the KCl concentration was raised to 55 mM to provide a "high K^+" buffer to induce secretion from the cells. In this case the NaCl concentration was lowered correspondingly to maintain isoosmolarity. When the experiments involved measurement of secreted products, all incubation buffers contained 40 μM eserine.

For measurement of the K^+-induced release of acetylcholine and dopamine, the cell culture medium was aspirated from cell-containing flasks, and after the cells were washed twice with 2 mL of low K^+ incubation buffer per flask, they were preincubated in 2.5 mL of the buffer for variable times as indicated under Results. The buffer was replaced with 2.5 mL of fresh incubation buffer containing either 6 mM KCl or 55 mM KCl, and the cells were further incubated for the desired time. Test compounds were generally present during both the preincubation and incubation periods. After incubation the buffer was collected and centrifuged at 750*g* for 5 min to pellet any cells dislodged during the incubation. For facilitation of removal of the remaining cells from the flasks, they were incubated in low K^+ incubation buffer that lacked Ca^{2+} but contained 0.05% trypsin and 0.02% EDTA. Samples of this cell suspension and the supernatant from the 750*g* centrifugation were taken for the measurement of acetylcholine, choline, and dopamine.

Uptake of Dopamine by Isolated Storage Granules. The storage granules and all drugs and other additives were contained in 0.15 M sucrose, 100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, and 10 mM potassium phosphate, pH 7.4. To 100-μL samples of a granule suspension (0.1 mg of protein) were added 10 μL of 13 mM ATP and 10 μL of test drug. Since the stock drug solutions were in ethanol, samples not receiving drugs received a corresponding amount of ethanol, which never exceeded 0.25%. After the addition of 10 μL of [^{3}H]dopamine (1 μCi), the samples were incubated at 37 °C for 5 min. The suspensions were placed on ice and mixed with 20 μL of a 15 mg/mL solution of dextran blue (M_r ~2 000 000), which served as a marker during the subsequent separation by Sephadex chromatography of the granules and their accumulated radioactivity from the remaining radioactivity. This separation procedure and the counting of the accumulated radioactivity were performed as described by Toll & Howard (1978). Uptake blanks, which were determined by incubation at 0 °C, were less than 10% of the total taken up at 37 °C. In all cases the amount of radioactivity associated with the granules at 0 °C was subtracted from the radioactivity associated with the granules at 37 °C to give a value for accumulated dopamine.

Retention of Dopamine and Acetylcholine by Isolated Granules. Storage granules and drugs were contained in the same buffer described for the uptake of dopamine into granules. To 100-μL samples of the granules (0.1 mg of protein) was added 10 μL of a solution of a test drug or a corresponding amount of ethanol. The granule suspension was incubated at 30 °C for 15 min or at 37 °C for 5 min as described under Results. The incubation was terminated by the addition of 0.9 mL of ice-cold incubation buffer containing 1 mM pargyline and 40 μM eserine. Samples were taken for the determination of dopamine and acetylcholine. Control studies demonstrated that, during the incubation, nonparticulate dopamine and acetylcholine were completely destroyed by contaminating monoamine oxidase and cholinesterase, respectively. Therefore, the retained dopamine and acetylcholine were measured in the incubated granule suspension without first separating the granules.

Determination of Acetylcholine and Choline. Extraction and determination of unlabeled acetylcholine and choline were as described by Freeman et al. (1975), except that the analysis was performed on a Hewlett-Packard 5710 A gas chromatograph equipped with a nitrogen detector. Trimethylacetylcholine perchlorate was used as an internal standard in all samples.

Newly synthesized [^{3}H]acetylcholine was separated from [^{3}H]choline by thin-layer chromatography after extraction of these compounds initially into 1 N formic acid–acetone (15:85) and subsequently into CH_2Cl_2 with dipicrylamine as described by Freeman et al. (1975). [^{14}C]Acetylcholine served as an internal standard. After the CH_2Cl_2 was dried under nitrogen, the acetylcholine was redissolved in acetone and spotted on

[1] Abbreviations used: DCCD, *N,N'*-dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; S-13, 5-chloro-*N*-*tert*-butyl-2'-chloro-4-nitrosalicylanilide.

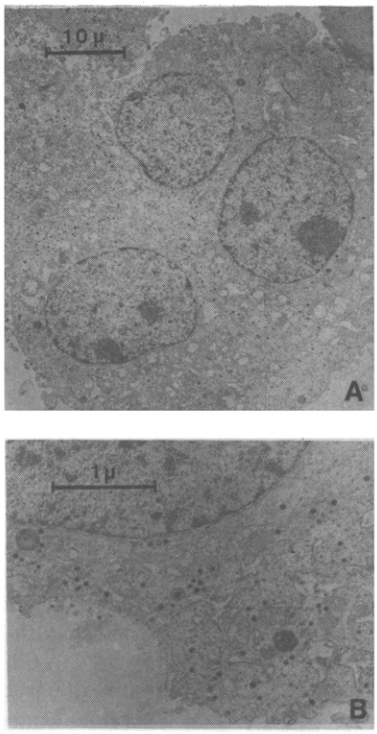

FIGURE 1: Electron micrograph of PC12 cells.

cellulose thin-layer plates. The plates were developed with a solvent system of ethylacetate–formic acid–H_2O (70:20:10). The spots were visualized with iodine and scraped into scintillation vials containing 0.5 mL of 0.1 N HCl. To each vial was added 10 mL of 3a70B liquid scintillation cocktail (Research Products International) and the radioactivity was counted by liquid scintillation spectrometry.

For measurement of newly synthesized acetylcholine, cells were preincubated in low K^+ incubation buffer for 15 min. The buffer was replaced with 5 mL of fresh buffer containing 2 μCi of [^{3}H]choline (84 Ci/mmol) per plate. After incubation for the desired time, the cells were washed twice with 5 mL of ice-cold incubation buffer containing 40 μM eserine per plate and once with 5 mL of 0.32 M sucrose and 40 μM eserine. The cells were scraped off the plate with a rubber policeman, and the plates were washed with an additional 1.5 mL of 0.32 M sucrose and 40 μM eserine. After the volume of the cell suspension was adjusted to 2.0 mL with 0.32 M sucrose and 40 μM eserine, a 0.4-mL sample was taken for measurement of acetylcholine. The remaining cell suspension was homogenized, and the granule fraction was prepared as described above.

Determination of Dopamine. All samples for the determination of dopamine were acidified to give a final concentration of 0.2 N $HClO_4$, 1 mM EGTA, and 10 mM dithiothreitol. Acidified samples from cells or cell particulate fractions were homogenized in a Polytron PT-10 (Brinkman Instruments Co.) and centrifuged at 5000*g* for 10 min at 4 °C. The supernatant was collected for the determination of dopamine by radioenzymatic assay. The method of Saller & Zigmond (1977) was used for the incubation of sample, *S*-adenosyl[^{3}H]methionine, and catechol *O*-methyltransferase, which was prepared from rat liver as described by Coyle & Henry (1973). The reaction was stopped and the reaction product 3-methoxytyramine was extracted as described by Peuler & Johnson (1977). 3-Methoxytyramine was purified by thin-layer chromatography and counted according to the method of Saller & Zigmond (1977), except that the thin-layer plates were scraped into scintillation vials containing 0.5 mL of 0.5 M sodium borate, pH 9.0. After vigorous shaking to elute the 3-methoxytyramine, the samples were mixed with 10 mL of 3a70B scintillation cocktail containing 2.5% bis(2-ethylhexyl)phosphoric acid.

Table I: Content of Dopamine and Acetylcholine in Whole Cells and in Subcellular Fractions of PC12[a]

fraction	dopamine	acetylcholine
whole cells	100 ± 12	100 ± 11
P_1	12 ± 1	3 ± 0.6
P_2	57 ± 2	32 ± 1
S_2	23 ± 3	53 ± 1

[a] P_1, P_2, and S_2 fractions were prepared as described under Materials and Methods. The results are expressed as the percentage of dopamine or acetylcholine found in whole cells, which contained 10.6 ± 1.2 nmol of dopamine per mg of protein and 3.8 ± 0.4 nmol of acetylcholine per mg of protein. The values are means ± SD for three samples.

Determination of ATP and Protein. ATP was measured as described by Nelson-Krause & Howard (1978), and protein was measured by the method of Lowry et al. (1951).

Results

Electron Microscopy of PC12 Cells. As shown in Figure 1, PC12 cells contain dense core granules that are similar in appearance to chromaffin granules of normal adrenal medullary cells but smaller in size and fewer in number. Note that in Figure 1A most of these granules are lined up along the plasma membrane. In fact the double rows of granules in adjacent cells almost delineate the plasma membranes that appear between the rows. This pattern of granules juxtaposed to plasma membranes was commonly but not invariably seen. A higher magnification of the granules is shown in Figure 1B. We did not observe translucent granules similar to those found in cholinergic nerve terminals. In PC12 cells acetylcholine appears to be contained in dense core granules; Schubert & Klier (1977) examined a homogenate of reserpine-treated PC12 cells and found that a sucrose gradient fraction enriched in acetylcholine-storing granules contained dense core granules and few other membrane structures.

Levels of Dopamine and Acetylcholine in PC12 Cells. In our hands PC12 cells contain 20 times as much dopamine as norepinephrine; thus, our examination of catecholamine metabolism in these cells has been limited to dopamine. Table I shows the levels of dopamine and acetylcholine in PC12 cells and in various fractions of cell homogenates. The recovery of these compounds in these fractions was 88–92%. The acetylcholine and dopamine in the S_2 fraction was nonparticulate in that these compounds failed to sediment when the S_2 fraction was centrifuged at 62000*g* for 40 min, a procedure sufficient to sediment synaptic vesicles isolated from rat brain (Toll & Howard, 1978).

Approximately 60% of the cell's dopamine and 35% of the acetylcholine are particulate and sediment in the mitochondrial fraction (P_2). Most of the dopamine and acetylcholine in the P_2 fraction was found to band in a sucrose density gradient at regions determined by Schubert & Klier (1977) to be enriched in granules and transmitter. Therefore, we have assumed that the bound acetylcholine and dopamine in the P_2 fraction are in storage granules and the P_2 fraction was used without further purification for most of the subsequent studies on isolated granules.

Dopamine Uptake by Isolated Granules. As shown in Table II, a storage granule fraction isolated from PC12 takes up dopamine by a system that operates in a medium of low Na^+

Table II: Dopamine Uptake by Isolated PC12 Granules[a]

additions	dopamine uptake[b]
control	100 ± 4
reserpine, 0.4 μM	33 ± 2
ouabain, 100 μM	90 ± 8
desipramine, 1 μM	96 ± 10
omit ATP	44 ± 1
FCCP, 2 μM	37 ± 1
S-13, 1 μM	33 ± 2
DCCD, 40 μM	46 ± 4
oligomycin, 2 μg/mL	101 ± 6

[a] The experiments were as described under Materials and Methods. [b] The results are expressed as the percentage of radioactivity taken up in control granules incubated without added reagents, and the values are means ± SD for duplicate incubations. The radioactivity taken up by control granules corresponded to 0.5 ± 0.02 pmol of dopamine per mg of protein.

and high K^+ composition and that is inhibited by reserpine but not by ouabain or desipramine. These results indicate that the uptake of dopamine is into the storage granules and not into contaminating plasma membrane fragments. Unlike the situation with the storage granules, transport of catecholamines across plasma membranes is Na^+ dependent, sensitive to ouabain, desipramine, and high K^+ concentration, and insensitive to reserpine (Carlsson et al., 1957, 1963; Euler et al., 1963; Colburn et al., 1968; Tissari et al., 1969; Horn et al., 1971; White & Paton, 1972; Koe, 1976; Paton, 1976).

Storage granules isolated from PC12 cells seem to retain newly accumulated catecholamine less readily than do storage granules isolated from bovine adrenal cells (data not shown). However, with respect to its bioenergetics, dopamine transport into isolated PC12 granules is quite similar to catecholamine transport into cell-free preparations of storage granules from adrenal medulla or brain. ATP stimulated dopamine uptake in PC12 granules about twofold (Table II). The uptake of dopamine was substantially inhibited by FCCP and S-13, which render membranes permeable to protons and thereby dissipate membrane potentials and transmembrane pH gradients (Slater, 1966; Hanstein, 1976). Dopamine uptake by the PC12 granules was also blocked by DCCD, which blocks catecholamine transport into chromaffin granules and synaptic vesicles by inhibiting the proton-translocating ATPase associated with these granules (Bashford et al., 1976; Toll et al., 1977). Dopamine uptake was not inhibited by oligomycin, which inhibits mitochondrial ATPase (Lardy et al., 1975) but not the ATPase or ATP-stimulated catecholamine transport activities of chromaffin granules and catecholamine-storing synaptic vesicles (Toll et al., 1977).

We have not obtained PC12 granules of sufficient purity to establish whether the granules contain a DCCD-sensitive ATPase. We have attempted to separate granules from mitochondria on a sucrose gradient after incubating a P_2 fraction with *p*-iodonitrotetrazolium violet to increase the buoyant density of the mitochondria as described by Davis & Bloom (1970). This more purified granule fraction, which was well separated from the bulk of the mitochondria, contained a DCCD-sensitive Mg^{2+}-ATPase; however, the ATPase activity was almost equally sensitive to oligomycin, suggesting that the fraction still was considerably contaminated with mitochondria or some other structure containing an oligomycin-sensitive ATPase.

Retention of Dopamine and Acetylcholine by Isolated PC12 Granules. Specific transport of acetylcholine into isolated synaptic vesicles has not been reported. We have found that isolated PC12 granules do not take up acetylcholine under the conditions described for Table II. Therefore, we have not been able to study the effect of metabolic inhibitors on the uptake of acetylcholine by isolated PC12 granules. However, we have examined their effects on the retention of endogenous acetylcholine by the granules.

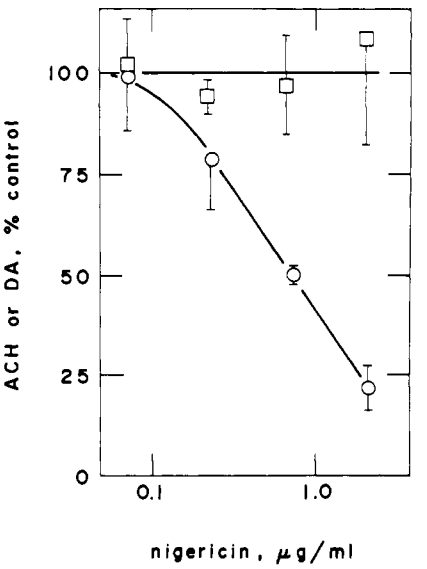

FIGURE 2: Effect of nigericin on the retention of dopamine (○) or acetylcholine (□) by PC12 granules. The granules were incubated for 15 min at 30 °C as described under Materials and Methods. The results are expressed as the percentage of dopamine or acetylcholine retained in control granules incubated without nigericin. The data points represent the means for duplicate incubations, and the error bars indicate the SD. Control granules retained 7.3 ± 1.3 nmol of dopamine per mg of protein and 5.3 ± 0.3 nmol of acetylcholine per mg of protein. Abbreviations used: AcCh, acetylcholine; DA, dopamine. The abscissa is a logarithmic scale.

Table III: Effect of Ionophores on the Retention of Dopamine and Acetylcholine by PC12 Granules[a]

treatment	dopamine	acetylcholine
control	100 ± 5	100 ± 11
valinomycin, 3 μg/mL	84 ± 6	91 ± 1
FCCP, 1.6 μM	95 ± 6	92 ± 2
valinomycin, 3 μg/mL, + FCCP, 1.6 μM	27 ± 3	91 ± 6

[a] The granules were incubated for 15 min at 30 °C as described under Materials and Methods. The results are expressed as the percentage of dopamine or acetylcholine retained in control granules incubated without added reagents, and the values are means ± SD for triplicate incubations. Control granules retained 19.6 ± 1 nmol of dopamine per mg of protein and 1.5 ± 0.2 nmol of acetylcholine per mg of protein.

Earlier studies showed that agents that dissipate the proton gradient across chromaffin granules and presumably synaptic vesicles cause an efflux of previously accumulated catecholamines from these vesicles. Proton ionophores dissipate this proton gradient, but they do so only with difficulty. This is because the initial efflux of protons from the granules establishes a diffusion potential that restricts additional efflux of protons. Proton ionophore induced efflux of protons is facilitated by treatments that allow an accompanying influx of some other cation to minimize the diffusion potential (Johnson & Scarpa, 1976b). Potassium ions made freely permeant by potassium ionophores can act as this other cation. Thus, nigericin, an ionophore that causes an H^+/K^+ exchange, or FCCP in combination with the K^+ ionophore valinomycin was more effective than FCCP alone at dissipating the proton gradient across chromaffin granules (Johnson & Scarpa, 1976b) and at decreasing the storage of catecholamines in

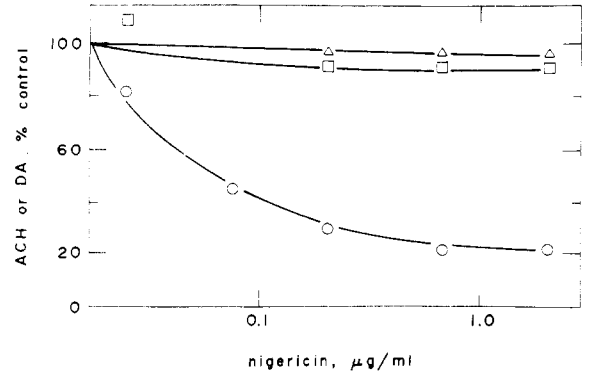

FIGURE 3: Effect of nigericin on the cellular levels of dopamine (○ and △) and acetylcholine (□). The cells were washed with cell incubation buffer and incubated as described under Materials and Methods for 5 min with 5 mL of fresh buffer containing varying concentrations of nigericin per plate. The buffer also contained 0.5 mM pargyline in the indicated cases (△), and for control cells it contained 0.1% ethanol, corresponding to the amount present in drug-containing buffers. After the incubation the cells were washed and harvested for the measurement of dopamine and acetylcholine. The results are expressed as the percentage of values in control cells which were incubated without nigericin. The data points represent the means for duplicate incubations. Control cells contained 5 ± 0.5 nmol of dopamine per mg of protein and 1.9 ± 0.1 nmol of acetylcholine per mg of protein. Abbreviations used: AcCh, acetylcholine; DA, dopamine. The abscissa is a logarithmic scale.

chromaffin granules and synaptic vesicles (Schuldiner et al., 1978; Toll & Howard, 1978).

Figure 2 and Table III show the retention of dopamine and acetylcholine in granules incubated in the presence or absence of these ionophores. In these experiments there was a considerable efflux of dopamine (up to 40%) from control granules during the incubation but we detected no efflux of acetylcholine from control granules during this time. The effects of the ionophores on the retention of dopamine in isolated PC12 granules resembled their effects on the retention of catecholamines by isolated synaptic vesicles from brain. While neither nigericin (Figure 2) nor FCCP plus valinomycin (Table III) substantially increased the efflux of endogenous dopamine from isolated PC12 granules, they did not appear to affect the ability of the granules to retain their endogenous stores of acetylcholine.

Studies on Intact Cells. We have examined whether the ionophores alter the storage of dopamine and acetylcholine in intact PC12 cells. Figure 3 shows that treatment of intact cells with nigericin for 5 min had little effect on the cellular level of acetylcholine, but the cellular level of dopamine was decreased substantially after the nigericin treatment. Most of the nigericin-induced decrease in dopamine level was prevented by pargyline, an inhibitor of monoamine oxidase.

The most reasonable explanation of the ability of pargyline to block the decrease in dopamine levels in nigericin-treated cells is that nigericin induces an efflux of dopamine from granules to a pool where it is oxidized by monoamine oxidase; pargyline inhibits this oxidation by monoamine oxidase. Consistent with this hypothesis is our finding that the granule fraction obtained from cells that had been previously treated with 0.2 μg of nigericin per mL for 5 min in the presence of 1 mM pargyline contained only 17% of the amount of dopamine present in granules obtained from control cells that had been treated with pargyline only.

The lack of a change in total acetylcholine content in cells treated with nigericin as described for Figure 3 masks the fact that nigericin does have some effect on granular stores of acetylcholine under these conditions. Granules isolated from cells that had been treated with nigericin (0.2 μg/mL) for 5 or 10 min contained 67–75% of the amount of acetylcholine found in granules from control cells. Since a minority of the cells' acetylcholine is granular (Table I), this loss of granular acetylcholine would not necessarily cause a detectable change in the total cellular level of acetylcholine.

Table IV: Effects of Ionophores on the Cellular Levels of Dopamine and Acetylcholine[a]

treatment	levels[b] dopamine	acetylcholine
control	100 ± 1	100 ± 12
valinomycin, 3 μg/mL	116 ± 6	99 ± 10
FCCP, 1.3 μM	121 ± 1	109 ± 13
valinomycin, 3 μg/mL, + FCCP, 1.3 μM	39 ± 4	106 ± 1

[a] The cells were washed with cell incubation buffer and incubated as described under Materials and Methods for 30 min with 5 mL of fresh buffer containing the indicated drugs per plate. The buffer for control cells contained 0.25% ethanol, corresponding to the amount present in drug-containing buffers. After the incubation the cells were washed with 5 mL of ice-cold buffer per plate and harvested for the measurement of dopamine and acetylcholine. [b] The results, which are expressed as the percentage of control values, are means ± SD for duplicate incubations. Control cells contained 4.6 ± 0.05 nmol of dopamine per mg of protein and 1.2 ± 0.14 nmol of acetylcholine per mg of protein.

Table V: K^+-Induced Release from PC12

conditions[a] K^+ (mM)	Ca^{2+} (mM)	% release[b] dopamine	acetylcholine	choline
6	1.3	1.0 ± 0.1	2.4 ± 0.3	33 ± 3
6	0	0.2 ± 0.04	0.9 ± 0.1	39 ± 3
55	1.3	17.7 ± 3.8	17.7 ± 0.7	47 ± 2
55	0	0.3 ± 0.05	1.3 ± 0.1	38 ± 1

[a] The cells were washed and incubated as described under Materials and Methods. The incubation was for 15 min in high K^+ buffer containing either no Ca^{2+} or 1.3 mM Ca^{2+}. [b] The results are expressed as the percentage of total cell dopamine, acetylcholine, or choline present in the incubation buffer at the end of the incubation. Values are means ± SD for triplicate incubations. Total cell acetylcholine, choline, and dopamine values were 1.9 ± 0.25, 2.2 ± 0.4, and 23.4 ± 2.1 nmol, respectively, per mg of protein.

Treatment of PC12 cells for 30 min with a combination of 1.3 μM FCCP and 3 μg of valinomycin per mL also caused a substantial decrease in the cellular level of dopamine (Table IV). The cellular level of acetylcholine was not significantly altered by this treatment.

The effects of nigericin or FCCP plus valinomycin on dopamine levels could not be attributed to a drop in cellular ATP secondary to an ionophore-induced inhibition of oxidative phosphorylation. Under the conditions reported for Figure 3 or Table IV, respectively, nigericin or a combination of FCCP and valinomycin caused less than a 15% decrease in cellular levels of ATP. This may be due to the fact that a major portion of the ATP in PC12 cells is derived from glycolysis rather than oxidative phosphorylation (our unpublished experiments).

Nigericin and the K^+-Induced Release of Dopamine and Acetylcholine. Since nigericin was found to reduce granule stores of dopamine more than it did granule acetylcholine, we examined whether nigericin would affect the K^+-induced release of dopamine more than the release of acetylcholine. This result would be expected if K^+ induces the release of these compounds from PC12 cells by an exocytosis process.

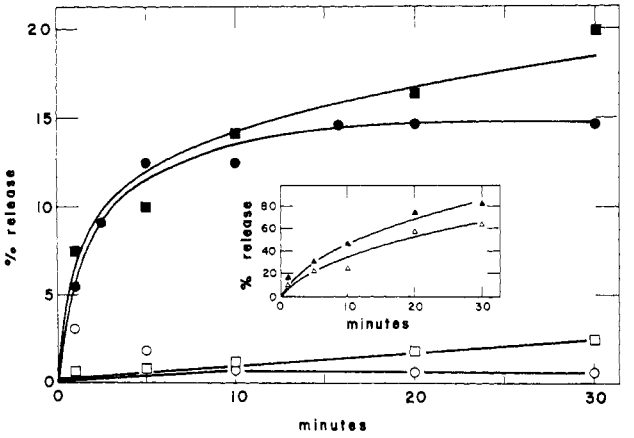

FIGURE 4: Time course of release of dopamine (○ and ●), acetylcholine (□, ■), and choline (insert, △ and ▲) from PC12 cells. The cells were washed and incubated for the indicated times as described under Materials and Methods. The incubation buffer contained 6 mM K^+ (○, □, and △) or 55 mM K^+ (●, ■, and ▲). The results are expressed as the percentage of total cell acetylcholine, choline, or dopamine present in the incubation buffer at the end of the incubation. The data points represent the means for triplicate incubations. Total cell acetylcholine, choline, and dopamine were 3.2 ± 0.9, 2.7 ± 0.8, and 9.7 ± 1.9 nmol, respectively, per mg of protein.

Table VI: Effect of Nigericin on the Release of Dopamine and Acetylcholine[a]

pretreatment[b]	K^+ for release[c] (mM)	% release[d] dopamine	% release[d] acetylcholine
control	6	2.2 ± 0.3	2.3 ± 0.9
	55	22.9 ± 1.5	12.1 ± 0.3
nigericin	6	9.2 ± 1.0	1.2 ± 0.2
	55	14.5 ± 1.7	3.6 ± 0.4

[a] The cells were washed and incubated as described under Materials and Methods. [b] Preincubation was for 5 min in a buffer containing 1 mM pargyline and either no nigericin or 0.5 μg of nigericin per mL. [c] Incubation was for 5 min in buffer containing 1 mM pargyline and the indicated concentration of K^+. [d] The results are expressed as the percent of total cell dopamine or acetylcholine present in the incubation buffer at the end of the incubation. Values are means ± SD for triplicate incubations. Total cell acetylcholine and dopamine values were 3.5 ± 0.6 and 18.7 ± 2.9 nmol, respectively, per mg of protein.

Figure 4 shows that 55 mM K^+ induced the release of both dopamine and acetylcholine. The K^+-induced release is Ca^{2+} dependent (Table V) and thus, in this regard, is similar to normal stimulated secretion of these compounds from other cells. A large amount of the cells' choline was also released during incubation in medium of either 6 mM K^+ or 55 mM K^+ (Figure 4 insert).

Table VI gives the effect of pretreatment with nigericin on the subsequent K^+-induced release of dopamine and acetylcholine from PC12 cells. The incubations were done in pargyline-containing medium to maintain cellular dopamine levels in the nigericin-treated cells (see Figure 3). Under these conditions there was an increased release of dopamine from nigericin-treated cells incubated in 6 mM K^+. We attribute this effect to a diffusion from increased cytosol pools of dopamine.

The nigericin pretreatment markedly decreased the amount of dopamine released from cells exposed to 55 mM K^+. The effect of nigericin is more noticeable if one compares the net induced release of dopamine (difference between release in 55 mM K^+ and 6 mM K^+) for control and nigericin-treated cells. Surprisingly, nigericin pretreatment also markedly inhibited the K^+-induced release of acetylcholine. No increased release of acetylcholine occurred during the period of pretreatment with nigericin. Therefore, the inhibition by nigericin of acetylcholine release cannot be attributed to a previous depletion of releasable acetylcholine stores caused by a nigericin-induced efflux of acetylcholine from the cells during the pretreatment period.

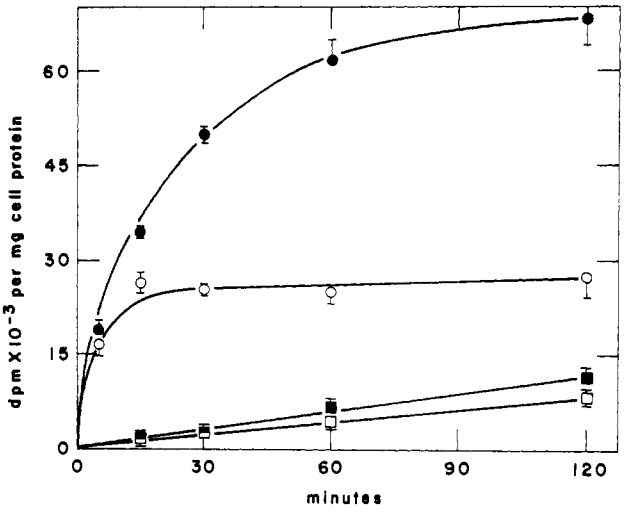

FIGURE 5: Time course of the synthesis of acetylcholine and its incorporation into granules. The cells were preincubated as described under Materials and Methods in 5 mL of cell incubation buffer per plate for 15 min and subsequently incubated for the indicated times in 5 mL of fresh buffer per plate containing 2 μCi of [^{3}H]choline (70 Ci/mmol) and in some cases (● and ■) 40 μM eserine. After the incubation the cells were washed 3 times with buffer and scraped from the plates into buffer containing 40 μM eserine. [^{3}H]Acetylcholine was measured in samples of whole cells (○ and ●) and a granule fraction (□ and ■) obtained from a cell homogenate. The data points represent the means for triplicate incubations, and the error bars indicate the SD.

Storage of Newly Synthesized Acetylcholine and Dopamine. We have not determined whether nigericin inhibits the K^+-induced release of dopamine and acetylcholine primarily by affecting the intracellular storage of these compounds or by some other mechanism. If the primary effect were on intracellular storage, our results suggest that releasable stores of acetylcholine are more sensitive to nigericin than are granular stores of acetylcholine as a whole. In other systems there is evidence that the granular storage and release of newly synthesized acetylcholine are different from those of older stores of acetylcholine (Collier, 1969; Richter & Marchbanks, 1971a,b; Zimmerman & Whittaker, 1977). Therefore, we have examined the effect of nigericin on the storage of newly synthesized acetylcholine in PC12 cells.

Newly synthesized acetylcholine was detected as [^{3}H]-acetylcholine formed when cells were incubated in a buffer containing [^{3}H]choline. Figure 5 shows the time course under these conditions of the appearance of [^{3}H]acetylcholine in cells and in the granule fraction of cell homogenates. The uptake of choline and its subsequent acetylation to acetylcholine were a rapid process. In the absence of the cholinesterase inhibitor eserine, a steady-state level of [^{3}H]acetylcholine was reached within 15 min. The level of [^{3}H]acetylcholine was much greater at all times measured when 40 μM eserine was present, and the [^{3}H]acetylcholine level was still gradually increasing at 2 h.

The appearance of [^{3}H]acetylcholine in the granule fraction followed a different pattern. The transport of acetylcholine into the granules appeared to be a relatively slow process, with the uptake linear with time up to 2 h, the longest time exam-

Table VII: Effect of Treatment of Cells with Nigericin or Iodoacetate on the Levels of Granular Acetylcholine and Cellular ATP[a]

	granular AcCh		
treatment	total AcCh	[^{3}H]AcCh	cellular ATP
control	100 ± 1	100 ± 12	100 ± 20
nigericin	45 ± 1	35 ± 11	88 ± 12
iodoacetate	107 ± 1	ND	31 ± 9

[a] Cells were preincubated as described under Materials and Methods in 5 mL of cell incubation buffer per plate for 15 min and subsequently incubated for 60 min in 5 mL of fresh buffer containing 2 μCi of [^{3}H]choline (84 Ci/mmol) per plate. The cells were then thoroughly washed with incubation buffer and incubated for an additional 10 min in 5 mL of incubation buffer with or without nigericin (0.2 μg/mL) or iodoacetate (0.5 mM). The nigericin-free buffers contained 0.1% ethanol to control for the ethanol present in the nigericin-containing buffer. After the final incubation the cells were scraped from the plates into buffer containing 40 μM eserine. Total acetylcholine and [^{3}H]acetylcholine were measured in samples of a granule fraction obtained from a cell homogenate. ATP was measured in extracts of unfractionated cells. The results, which are expressed as the percentage of control values, are the means ± SD for triplicate incubations. Control granular acetylcholine values were 2.5 ± 0.2 nmol/mg of protein and 23 300 ± 1524 dpm/mg of protein. Control cellular ATP was 14.8 ± 3 nmol/mg of protein. Abbreviations used: AcCh, acetylcholine; ND, not determined.

ined. Furthermore, there was only a small increase in the amount of [^{3}H]acetylcholine found in granules when the incubation was performed in the presence of eserine. This, in part, reflected the fact that eserine also increased the level of unlabeled acetylcholine in the cells (data not presented) so that the specific activity of the cytosol pool of acetylcholine was affected little by eserine.

Table VII gives evidence that in intact cells nigericin can cause a substantial efflux from granules of previously accumulated acetylcholine. For these experiments cells were incubated with [^{3}H]choline for 60 min, washed, and further incubated in the presence or absence of nigericin (0.2 μg/mL). After 10 min the levels of total acetylcholine and [^{3}H]-acetylcholine were determined for the granule fraction of a cell homogenate. After nigericin treatment only 35% of the [^{3}H]acetylcholine and 45% of the total acetylcholine remained in the granule fraction. Therefore, newly synthesized acetylcholine in the granules was not significantly more sensitive to nigericin than granule stores of acetylcholine as a whole.

Note that nigericin caused a greater efflux of acetylcholine from granules under the conditions described for Table VII than under the conditions used for Figure 3. Presumably, the prolonged exposure to incubation buffer in the experiments of Table VII increases the effect of nigericin on granular storage of acetylcholine in intact cells. However, this incubation condition does not change the resistance to nigericin of acetylcholine in isolated granules. Granules were isolated from cells that had been incubated with [^{3}H]choline for 60 min as described for Table VII. The isolated granules, which contained [^{3}H]acetylcholine, were then treated with 0.2 μg of nigericin per mL for 10 min at 37 °C. Under these conditions nigericin caused no loss of [^{3}H]acetylcholine from the granules. Thus, nigericin caused an efflux of acetylcholine from granules in intact cells but not from isolated granules.

We do not believe that nigericin causes an efflux of acetylcholine from granules in intact cells by inhibiting oxidative phosphorylation in mitochondria and thereby lowering cellular ATP levels. As shown in Table VII, iodoacetate caused a much greater decrease in the cellular ATP level than did nigericin, but iodoacetate did not cause an efflux of acetylcholine from granules in intact cells.

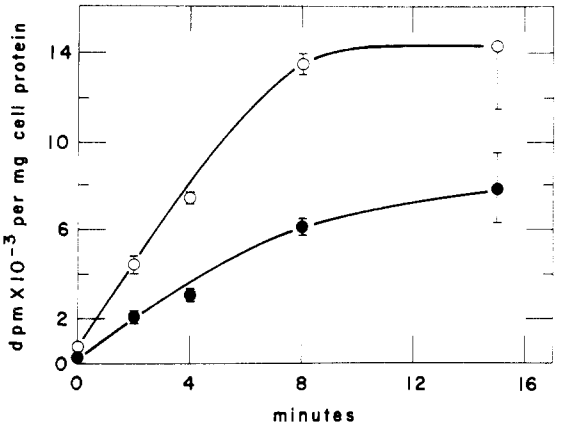

FIGURE 6: Cellular uptake of dopamine and its accumulation in granules. The cells were washed twice with 5 mL of incubation buffer per plate and incubated as described under Materials and Methods in 5 mL of fresh incubation buffer per plate containing 1 μCi of [^{3}H]dopamine (33 Ci/mmol) for the indicated times. The cells were then washed twice with 5 mL of ice-cold incubation buffer and once with 5 mL of 0.32 M sucrose and 10 mM sodium phosphate, pH 7.4. The cells were harvested, and the radioactivity was counted in a sample of the total cell homogenate (○) and in a granule fraction (●) obtained from the homogenate. The radioactivity was counted by scintillation spectrometry. Over 90% of the accumulated radioactivity was associated with dopamine, as determined by thin-layer chromatography. The data points represent the means for triplicate incubations, and the error bars indicate the SD.

Loading of Dopamine into Granules in Intact Cells. We have found that, unlike the case with acetylcholine, newly accumulated or synthesized dopamine is rapidly loaded into granules in PC12 cells. As shown in Figure 6, cells incubated in buffer containing [^{3}H]dopamine rapidly accumulated a substantial amount of labeled dopamine. Figure 6 also shows that ~50% of the newly accumulated dopamine was loaded into granules soon after entering the cell.

Figure 7 shows the fate of newly synthesized [^{3}H]dopamine in cells incubated in the presence of the precursor [^{3}H]tyrosine. A significant portion of the labeled tyrosine that entered the cells was converted to dopamine, approximately half of which was soon thereafter loaded into granules. These studies demonstrate that, unlike the case with acetylcholine, newly accumulated or synthesized dopamine is rapidly loaded into granules in PC12 cells.

Discussion

In our studies we have assumed that each PC12 cell stores both dopamine and acetylcholine, but in different granules. This assumption seems warranted because fluorescence microscopy has shown that all the cells contain a rich content of catecholamines (our unpublished experiments); therefore, at least some of these cells must also store acetylcholine. Schubert & Klier (1977) concluded that dopamine and acetylcholine are stored in different granules because dopamine-containing granules have a buoyant density different from that of acetylcholine-containing granules.

All of our results support the hypothesis that the transport of catecholamines into storage granules is driven by a transmembrane proton gradient that is established by the activity of a proton-translocating ATPase in the granules. Dopamine transport into PC12 granules was inhibited by DCCD, which inhibits proton-translocating ATPases, or by ionophores that dissipate proton gradients. Furthermore, these ionophores also produced a rapid efflux of catecholamines from PC12 granules. In these respects the dopamine-storing granules of PC12 cells resemble chromaffin granules purified

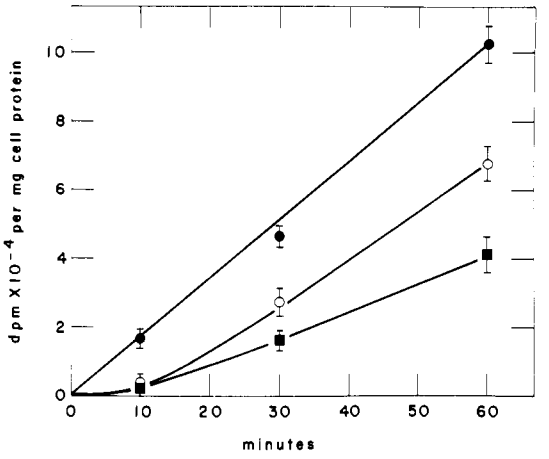

FIGURE 7: Time course of the synthesis of dopamine and its incorporation into granules. The cells were washed, incubated, and harvested as described in the legend to Figure 6 except that the incubation buffer contained 0.4 μCi/mL [^{3}H]tyrosine (40 Ci/mmol). A granule fraction was obtained from a portion of the harvested cells. A pelleted sample of the whole cells and the granule fraction were each resuspended in 3 mL of dilute acidic acid, pH 4.0. [^{14}C]Dopamine (0.1 μCi) was added to the suspensions as an internal standard, and they were homogenized with a Polytron PT-10. After centrifugation at 20000g for 20 min, one sample of the supernatant was taken for the measurement of total ^{3}H and another sample was taken for the measurement of [^{3}H]dopamine. The second sample (2 mL) was added to 300 mg of heat-activated alumina and brought to a volume of 5 mL with dilute acetic acid, pH 4.0. While the mixture was stirred, the pH was raised to 8.5 with several drops of 1 N NH_4OH. The alumina was transferred to a small column and washed with 4 mL of H_2O, and the dopamine was eluted with 2 mL of 0.3 N acetic acid. The eluate was lyophilized to dryness, redissolved in acidic methanol, pH 4.0, and spotted on a cellulose thin-layer plate. The plate was developed in butanol–water–acetic acid (25:10:4), and the radioactivity in the dopamine spot was counted by scintillation spectrometry. The data points represent the means for triplicate incubations, and the error bars indicate the SD. (●) Total radioactivity in cells; (○) [^{3}H]dopamine in cells; (■) [^{3}H]dopamine in granules.

from bovine adrenal medulla or catecholamine-storing synaptic vesicles from rat brain.

An important finding in the present studies was that various ionophores produced the same effects on the granular storage of dopamine in intact cells as they did on dopamine storage in isolated granules, in vitro. Thus, the interference of these ionophores with granular storage of dopamine in intact cells was likely due to a primary effect of the granules themselves rather than being secondary to an effect of the ionophores on some other cell organelle.

One of the ionophores, nigericin, also affected the granular storage of acetylcholine, and it is tempting to attribute the nigericin effects to a dissipation by nigericin of some transgranular ion gradient that drives acetylcholine transport into the granules. However, other studies are required to elucidate the mechanism with certainty. At present we do not understand why nigericin causes an efflux of acetylcholine from granules in intact cells while acetylcholine storage in isolated granules is unaffected by nigericin. It may be that in intact cells acetylcholine in granules normally turns over rapidly and nigericin appears to cause an efflux of acetylcholine by just inhibiting loading of acetylcholine into the granules. This explanation seems unlikely because some compounds, e.g., iodoacetate, inhibit the loading of acetylcholine into granules without affecting granule stores of previously accumulated acetylcholine (L. Toll and B. D. Howard, unpublished experiments). Alternatively, nigericin may not exert its action on the acetylcholine-storing granules directly but rather act indirectly by a primary effect on some other organelle in the cells. We cannot exclude such a mechanism, but the effect of nigericin on granule stores of acetylcholine is not secondary to an inhibition by the ionophore of mitochondrial ATP synthesis; iodoacetate, which caused a greater decrease in cellular ATP than did nigericin, did not induce an efflux of acetylcholine from granules in intact cells.

An interesting possibility is that acetylcholine-storing granules become susceptible to nigericin only when they interact with some particular component of the cell. For example, the granules may have to interact with a special intracellular site in order for acetylcholine loading to occur, and nigericin could exert its effect on granule stores of acetylcholine only during that interaction. The fact that the loading of acetylcholine into granules lagged considerably behind its synthesis is consistent with acetylcholine loading occurring at special intracellular sites that are sufficiently limited and saturated to make the rate of acetylcholine loading slow.

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Nuclear Relaxation Studies of the Interaction of Substrates with a Metalloaldolase from Yeast†

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ABSTRACT: The essential Zn^{2+} of yeast aldolase can be replaced by Mn^{2+} to yield an active paramagnetic holoenzyme [Kobes, R. D., Simpson, R. T., Vallee, B. L., & Rutter, W. J. (1969) *Biochemistry 8*, 585] which causes enhanced relaxation rates of substrate nuclei [Mildvan, A. S., Kobes, R. D., & Rutter, W. J. (1971) *Biochemistry 10*, 1191]. The frequency dependencies of the paramagnetic effects of aldolase–Mn^{2+} on the nuclear relaxation rates of the substrate acetol phosphate have been studied by ^1H NMR (at 100, 220, and 360 MHz), ^{31}P NMR (at 24.3, 40.5, 72.9, and 145.8 MHz), and ^{13}C NMR (at 25.1 MHz) using 90% enriched [2-^{13}C]acetol phosphate. Active-site binding of acetol phosphate in the NMR experiments was indicated by its displacement from the paramagnetic complex by the linear competitive inhibitor hexitol bisphosphate. The kinetic parameters of the exchange of acetol phosphate out of the ternary complex ($k_{off} = 1.1 \times 10^5$ s^{-1}, $E_{act} = 9.5$ kcal/mol, $k_{on} = 6.1 \times 10^7$ M^{-1} s^{-1}) determined from the temperature and frequency dependencies of $1/T_{2p}$ of ^{31}P indicated that the ternary complex is kinetically competent to be a catalytic intermediate. The correlation time for the Mn^{2+}–acetol phosphate dipolar interaction on aldolase was estimated as 2.2 ± 1.1 ns by the frequency dependence of $1/T_{1p}$ of the protons and ^{31}P of acetol phosphate, as well as that of water protons. Distances from the enzyme-bound Mn^{2+} to the methyl (9.3 Å) and methylene protons (9.5 Å) and the phosphorus (7.9 Å) of acetol phosphate, as well as to the carbonyl carbons of [2-^{13}C]acetol phosphate and [2-^{13}C]dihydroxyacetone phosphate (7.6 Å), are too great by 4.8 ± 0.6 Å for direct carbonyl coordination by the Mn^{2+}, although the conformation of bound acetol phosphate indicates that the carbonyl group points toward the Mn^{2+}. In contrast, the binary Mn–acetol phosphate complex in the absence of aldolase shows direct phosphate coordination with a Mn^{2+}–phosphorus distance of 2.9 Å. The use of Co^{2+}, a paramagnetic probe appropriate for shorter distances due to its smaller effective magnetic moment, yielded Co^{2+} to ^{31}P distances in the ternary aldolase–Co^{2+}–acetol phosphate complex consistent with that found with Mn^{2+}. While this outer sphere Co^{2+} complex was found to be kinetically competent, the existence of an inner sphere Co^{2+} complex could not be demonstrated. Hence, if the metal participates in catalysis by polarizing the carbonyl group to stabilize an enolate intermediate, it must do so through an intervening ligand. The magnitude of the Mn^{2+}–substrate distances on aldolase together with the observation of little or no change in the number of fast-exchanging water ligands on Mn^{2+} when substrates bind ($q \sim 1$) argues against an intervening water ligand. The Mn^{2+}–substrate distances are appropriate for an intervening imidazole ligand which would effectively transmit the electrophilic effect of the metal to the carbonyl group of the substrate through a hydrogen bond.

Fructose-1,6-bisphosphate aldolases (EC 4.1.2.13) from a number of sources have been divided into two mechanistic classes (Rutter, 1964). Class I aldolases, isolated from animals, higher plants, and certain bacteria, have been found to be tetrameric proteins of 150000 molecular weight (Kawahara & Tanford, 1966). Horecker and co-workers (Grazi et al., 1962; Horecker et al., 1963) showed that the reaction catalyzed by class I aldolases from muscle proceeds through a Schiff base intermediate involving an active-site lysine residue. In contrast to the class I aldolases, the class II aldolases, isolated from bacteria and fungi, apparently do not employ a Schiff base intermediate for catalysis (Horecker et al., 1963; Kobes et al., 1969). These enzymes have been found to contain one essential zinc ion per subunit (Richards & Rutter, 1961). The class II aldolases also differ in gross structure from the class I enzymes, existing as dimers with molecular weights near 80000 (Harris et al., 1969). The yeast enzyme, the prototype of class

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