Influence of Histone Phosphorylation upon Histone–Histone Interactions Studied in Vitro†

Jan Szopa,* Geert Jacob, and Hans-Adolf Arfmann

ABSTRACT: Histones H2b and H3, phosphorylated in vitro with the catalytic subunit of protein kinase I from rabbit skeletal muscle, were used to estimate the influence of histone phosphorylation upon histone–histone complex formation. Stoichiometry and interaction affinity of the complexes H2a–H2b, H4–H2b, and H4–H3 were determined by using the continuous variation method based on circular dichroism or fluorescence intensity. All complexes exhibit a 1:1 stoichiometry in sodium phosphate or sodium chloride solution of pH 7.0. The association constants of the complexes containing phosphorylated H2b were only slightly reduced, whereas that with phosphorylated H3 was strongly reduced relative to those of the nonphosphorylated species.

The basic subunit of chromatin, the nucleosomal core particle, is now a well-defined unit. It contains two of each of the histones H2a, H2b, H3, and H4, which form a protein core by specific interactions; the DNA is wrapped around that core (Kornberg, 1977). In the living cell the histone–histone and also the histone–DNA interactions within the nucleosome are modulated by various posttranslational modifications, like acetylation and phosphorylation. The phosphorylation of histones has been followed during the cell cycle of various cell types (Bradbury et al., 1974; Gurley et al., 1978), and a superphosphorylation of H1 and a phosphorylation of H3 have been reported to parallel the chromatin condensation in mitosis (Gurley et al., 1978). From in vitro studies with isolated core particles H3 was found to be the major phosphate acceptor (Shoemaker & Chalkley, 1978; Arfmann & Bode, 1979).

The specificity of histone–histone interactions can be followed in vitro by probing the complex formation of all possible pairwise combinations. D'Anna & Isenberg (1974a) reported strong interactions only for the histone pairs H2a–H2b, H2b–H4, and H3–H4. Up to now the influence of a histone modification upon the strength of histone–histone complex formation has not been investigated. We used in vitro phosphorylated histones H2b and H3 to estimate the stoichiometry and association constants for the three histone pairs which form strong complexes in the unmodified form. For phosphorylation the catalytic subunit of cAMP-dependent protein kinase from rabbit muscle was applied. This is a cytoplasmic enzyme (Beavo et al., 1975), and the phosphorylation sites may not be the same as with nuclear enzymes. There is, however, evidence (Jungmann et al., 1975), though controversial (Zick et al., 1979), which indicates a translocation of cAMP-dependent enzymes into the nucleus. On the other hand, the sites of phosphate incorporation into H2b by the above-mentioned enzyme are known (Yeaman et al., 1977), and the site of H3 has been determined very recently in our laboratory (H.-A. Arfmann and K.-H. Plank, unpublished experiments). Hence, the influence of histone phosphorylation on the interaction affinity can be discussed with reasonable molecular imagination.

Materials and Methods

Whole histone from calf thymus was prepared from fresh thymus glands by the method of Kobayashi & Iwai (1970). Resolution of whole histone into its components was achieved by hydrophobic chromatography (Arfmann & Shaltiel, 1976), and coeluting H2b and H4 were further resolved by chromatography on Bio-Gel P60 (Böhm et al., 1973). The histone fractions characterized by polyacrylamide slab gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (Laemmli, 1970) were free of impurities.

The phosphorylation was performed with the catalytic subunit of cAMP-dependent protein kinase from rabbit skeletal muscle, which was purified according to Beavo et al. (1974). Twenty milligrams of histone H3 or H2b was dissolved in 20 mL of incubation buffer (Kurochkin et al., 1977), containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.3 mM EGTA, and 0.3 mM ATP; 0.5 mL of kinase solution (500 μg of protein) was added, and the mixture was incubated for 5 h at 37 °C. The whole mixture was applied on a Bio-Gel P2 column (2.5 × 32 cm), which had been equilibrated with 10 mM HCl; the histones were eluted with the same solution. The absorbance at 230 nm was followed, and appropriate histone fractions were pooled and lyophilized.

The purity of the phosphorylated histone fractions was established by gel electrophoresis as described above. The phosphate content was estimated by the method of Ames (1966). For histone H2b ~2.4 mol of phosphate and for histone H3 between 0.9 and 1.2 mol of phosphate per mol of histone were determined.

For determination of histone–histone complex formation, stock solutions of the individual histones were prepared at 0.1 mM in water (for H3, see below). The histone concentrations were estimated from the absorbance at 230 or 275 nm. Molar extinction coefficients at 230 nm for H2a, H2b, and H4 were taken as 43, 54, and 42 cm^{-1} mM^{-1}, respectively (D'Anna & Isenberg, 1974a,b). At 275.5 nm, molar extinction coefficient values of 4.05, 6.7, 4.04, and 5.4 cm^{-1} mM^{-1} were taken for H2a, H2b, H3, and H4, respectively (D'Anna & Isenberg, 1974b). Histone H3 was reduced (Ruiz-Carrillo & Allfrey, 1973) and chromatographed on Sephadex G-25 superfine equilibrated with 10 mM HCl and 1 mM dithiothreitol; H3 was eluted with the same solution, lyophilized, and dialyzed against the interaction buffer (see below) in the presence of 1 mM dithiothreitol.

Series of solutions for the continuous variation method were prepared from stock solutions of histones; the sum of concentrations of the two histones after dilution in each series was constant (5–9 μM). The solutions were diluted with sodium phosphate buffer or sodium chloride in 5 mM cacodylate

† From the Gesellschaft für Biotechnologische Forschung, Abteilung Molekularbiologie, D-3300 Braunschweig-Stöckheim, Federal Republic of Germany. *Received August 24, 1979.* This work was supported by the Deutsche Forschungsgemeinschaft (Wa 91); J.S. is supported by a grant from the Alexander von Humboldt-Stiftung.

0006-2960/80/0419-0987$01.00/0

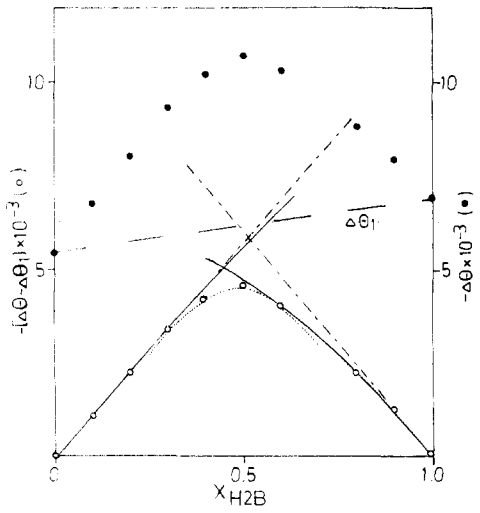

FIGURE 1: Treatment of the experimental data. In the case of CD measurements, the molar ellipticity at 220 nm was extracted from the spectra and corrected for the solvent ($\Delta\theta$) (●). The abscissa indicates the mole fraction of one histone component (H2b) in the mixture with the partner component (H2a); the total histone concentration was kept constant (8.8 μM). From the pure components (mole fraction 0 and 1) the spectral changes of the noninteracting histone mixture ($\Delta\theta_1$) can be deduced. The difference $\Delta\theta - \Delta\theta_1$ (○) is used for further calculation [cf. also D'Anna & Isenberg (1973b)]. As described under Materials and Methods, parabolas (—) and tangents (- - -) at mole fractions 0 and 1 were calculated and the theoretical and experimental maximum values at mole fraction 0.5 were determined; these data allow the calculation of the degree of dissociation and the equilibrium constant.

buffer, both of pH 7.0; the final concentration of sodium phosphate was 16 mM and sodium chloride 0.20 M.

CD spectra were recorded on a Roussel-Juan 185 Model II dichrograph; cuvettes of 10-mm path length were used. The ellipticity was extracted at 220 nm to plot the continuous variation curves. The CD spectra of the phosphorylated histones were the same as those of the nonphosphorylated species. Fluorescence intensity was measured with a Schoeffel type RRS 1000 instrument and 1-cm cuvettes. Excitation was at 279 nm, and emission was monitored at 325 nm. The measurements were performed at 20 °C.

The data were analyzed by using the old graphical method (Schaeppi & Treadwell, 1948) and a modification of it (Figure 1). This procedure was done by a small computer (calculator TI-59 from Texas Instruments) by fitting parabolas through the first and last four values, respectively, including the zero points, and by calculating the tangents at the starting points in order to reduce the arbitrariness of drawing tangents at the starting points of complex formation. This allows the calculation of the coordinates of the theoretical maximum value (E_0) for complete formation. The experimental maximum value (E) was determined by the same procedure by fitting a parabola through the points around the mole fraction of 0.5.

From the values E_0 and E taken at the mole fraction of 0.5 the degree of dissociation (α) and the equilibrium constant (K) can be derived. It is easily shown that the quotients $E_0 - (E/E_0)$ and E/E_0 correspond to α and $1 - \alpha$, respectively. The association constants for a dimeric (M^{-1}) and a tetrameric (M^{-3}) complex can be calculated with the equations

$$K_2 = \frac{[AB]}{[A][B]} = \frac{1}{c}\frac{1-\alpha}{\alpha^2}$$

$$K_4 = \frac{[A_2B_2]}{[A]^2[B]^2} = \frac{1}{2c^3}\frac{1-\alpha}{\alpha^4}$$

Table I: Apparent Association Constants (K_a) of Histone–Histone Interaction Determined by the Continuous Variation Method

histone pair	buffer	total histone concn (μM)	K_a (μM^{-1}) from CD data	K_a (μM^{-1}) fluorescence intensity
H2b–H4	16 mM phosphate	8.4	1.1	
H2b(P)–H4	16 mM phosphate	8.4	0.55	
H2a–H2b	16 mM phosphate	4.4	3.6	
	16 mM phosphate	8.8	3.3	
	0.2 M NaCl	4.4	3.3	
	0.2 M NaCl	8.8	3.1	2.8
H2a–H2b(P)	16 mM phosphate	4.4	0.34	
	16 mM phosphate	8.8	0.31	
	0.2 M NaCl	4.4	0.29	
	0.2 M NaCl	8.8	0.25	
H3(P)–H4	16 mM phosphate	6	0.021[a]	0.026[a]
	16 mM phosphate	8.6	0.019[a]	

[a] Apparent association constants (in μM^{-3}) estimated for the formation of tetrameric complex $(H3H4)_2$. For the respective unphosphorylated species D'Anna & Isenberg (1974a) determined an association constant of 700 μM^{-3}.

c is the input concentration of component A (or B) at the mole fraction of 0.5.

Results and Discussion

In a series of investigations with the continuous variation method, D'Anna & Isenberg (1974a) established the range of interaction strength of the possible pairs of the inner histones. They deduced 1:1 stoichiometry, but high affinity only for H2a–H2b, H3–H4, and H2b–H4 interaction. In order to estimate the influence of histone phosphorylation upon the formation of these high-affinity histone complexes, we decided to use phosphorylated H2b and H3. These two histones are good substrates for cAMP-dependent protein kinase from rabbit muscle, and, furthermore, the positions of phosphate incorporation into H2b have been established recently (Jungmann et al., 1975) while those in H3 have been determined in our laboratory (H.-A. Arfmann and K.-H. Plank, unpublished experiments). H2b is phosphorylated by this enzyme at the serine residues 32 and 36; these are also the main sites of several other cAMP-dependent kinases (Johnson, 1977). Histone H3 is phosphorylated at the serine residue 10.

Figures 2–4 show continuous variation curves comparing nonphosphorylated and phosphorylated H2b and H3 with the strong interacting partners H2a and H4, respectively. The histone concentration applied was in the range of 4–9 μM; the interaction was determined from changes either in the CD spectrum or in the tyrosine fluorescence in 16 mM sodium phosphate buffer (D'Anna & Isenberg, 1973a) or in 0.2 M NaCl, both at pH 7. The extracted results are listed in Table I.

Table I reveals that the interaction parameters are the same when determined in 16 mM sodium phosphate or 0.2 M NaCl and are also independent from the method of observation used. There is furthermore only a slight dependence upon the histone concentration. With unphosphorylated histones H3–H4 at the concentration (8.4 μM) applied, complex formation is complete; hence, an affinity constant could not be derived. For comparison with the phosphorylated H3–H4 pair we rely on the value determined by D'Anna & Isenberg (1974a) at lower histone concentration of 700 μM^{-3} for the formation of a tetrameric complex. This is justified as our affinity constants obtained for the other nonphosphorylated histone pairs are in

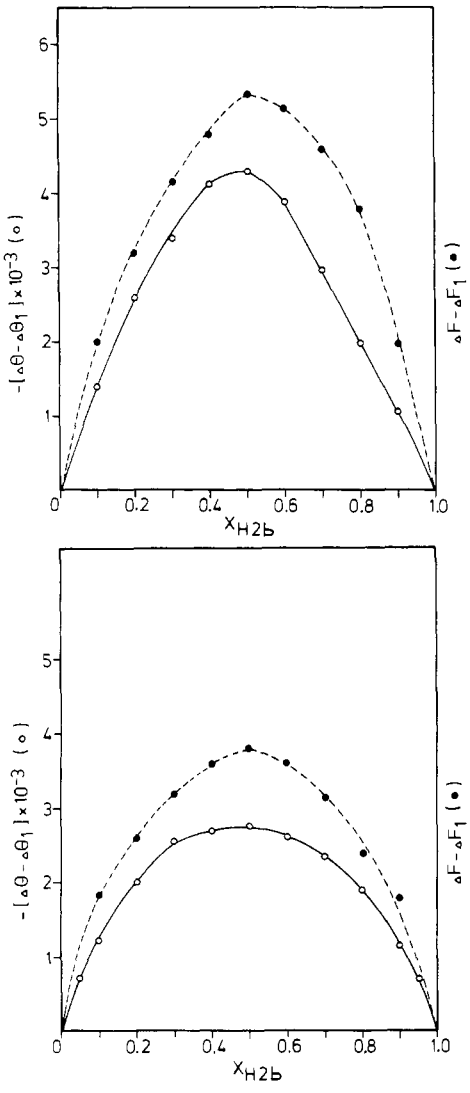

FIGURE 2: Continuous variation plots for the interaction of H2a with nonphosphorylated H2b (top) and phosphorylated H2b (bottom) based on the molar ellipticity at 220 nm (Θ) and on the fluorescence intensity at 325 nm (F), respectively. The treatment of the experimental data is described in the legend of Figure 1 and under Materials and Methods. In the case of fluorescence measurements, ΔF and ΔF_1 (in arbitrary units) refer to the fluorescence intensity of the interacting and noninteracting histone mixture, respectively. The total histone concentration was 8.8 μM. The measurements were performed at 20 °C in 0.2 M NaCl and 5 mM sodium cacodylate of pH 7.

good agreement with those published by D'Anna & Isenberg (1974a).

The results in Table I for the phosphorylated histone pairs first indicated a 1:1 stoichiometry as found with the nonphosphorylated species. However, with all three histone pairs investigated the interaction affinity decreases with histone phosphorylation. This decrease is rather graduated, being smallest with H2b–H4 (1.7 kJ/mol), more significant with H2a–H2b (6 kJ/mol), and largest with H3–H4 (25 kJ/mol). The latter value refers to a tetrameric complex; however, considering half of it (12.5 kJ/mol) for a comparison with the other dimeric complexes, this decrease is still significant.

The phosphates incorporated into H2b and H3 are in both cases located in the basic N-terminal parts of the histones which have less tertiary structure and are believed to interact with the nucleosomal DNA (Bradbury & Crane-Robinson, 1971). Obviously, phosphorylated serine residues interfere with histone–histone interaction even in this part of the molecule. However, a twofold phosphorylated H2b at positions between

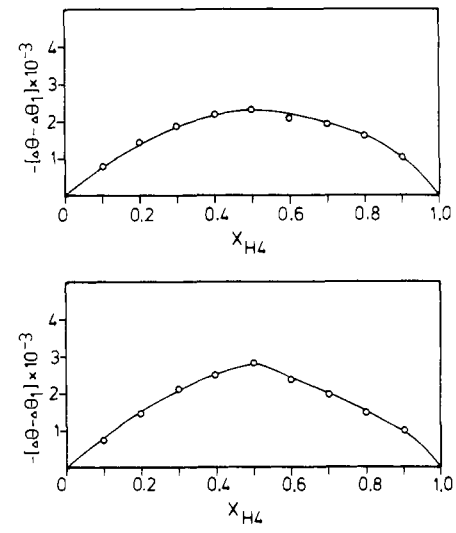

FIGURE 3: CD continuous variation plots for the interaction of H4 with nonphosphorylated H2b (bottom) and phosphorylated H2b (top). For an explanation of the indicated terms, see the legend of Figure 1. Total histone concentration was 8.4 μM; the experiments were performed at 20 °C in 16 mM sodium phosphate of pH 7.

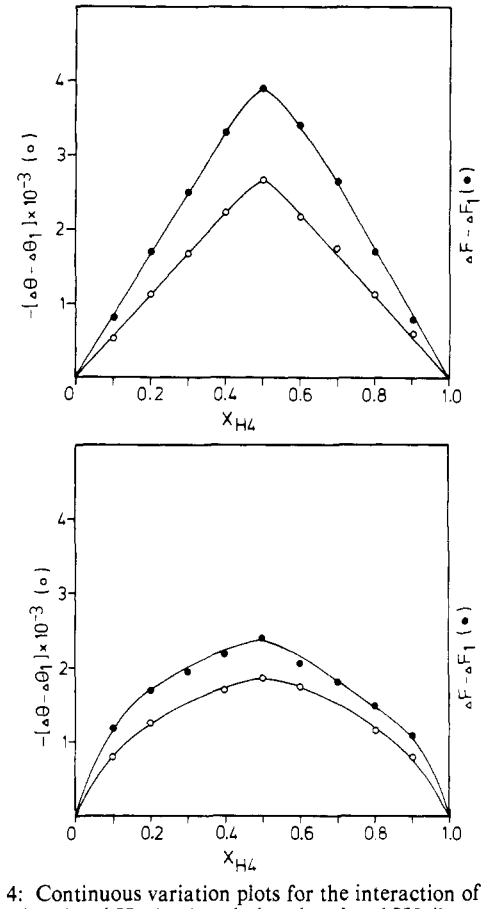

FIGURE 4: Continuous variation plots for the interaction of H4 with nonphosphorylated H3 (top) and phosphorylated H3 (bottom). For an explanation of the indicated terms, see the legends of Figures 1 and 2. As histones H3 and H4 alone undergo slow conformational changes, the spectral changes were determined by extrapolation to time zero after addition of the salt (D'Anna & Isenberg, 1972, 1973b). The total histone concentration was 6 μM; the experiments were performed at 20 °C in 16 mM sodium phosphate of pH 7.

30 and 40 reveals only a small decrease in interaction affinity with H4 and also with H2a, whereas H3 phosphorylated once at position 10 shows a rather strong decrease. This again reflects the hierarchy of histone–histone recognitions in the

formation of the nucleosome.

Acknowledgments

We thank Professor K. G. Wagner for helpful consultations. We are very grateful to R. Jähne for typing and to E. Kühne for drawing the figures.

References

Ames, B. N. (1966) *Methods Enzymol. 8*, 115-118.
Arfmann, H.-A., & Shaltiel, S. (1976) *Eur. J. Biochem. 70*, 269-274.
Arfmann, H.-A., & Bode, J. (1979) *Int. J. Biol. Macromol.* (in press).
Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1974) *Methods Enzymol. 38c*, 299-308.
Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1975) *Adv. Cyclic Nucleotide Res. 5*, 241-251.
Böhm, E. L., Strickland, W. N., Strickland, M., Thwaits, B. H., van der Westhuizen, D. R., & von Holt, C. (1973) *FEBS Lett. 34*, 217-221.
Bradbury, E. M., & Crane-Robinson, C. (1971) in *Histones and Nucleohistones* (Phillips, D. M. P., Ed.) pp 85-134, Plenum Press, London and New York.
Bradbury, E. M., Inglis, R. J., & Matthews, H. R. (1974) *Nature (London) 247*, 257-261.
D'Anna, J. A., Jr., & Isenberg, I. (1972) *Biochemistry 11*, 4017-4025.
D'Anna, J. A., Jr., & Isenberg, I. (1973a) *Biochemistry 12*, 1035-1043.
D'Anna, J. A., Jr., & Isenberg, I. (1973b) *Biochemistry 12*, 4987-4992.
D'Anna, J. A., Jr., & Isenberg, I. (1974a) *Biochemistry 13*, 4992-4997.
D'Anna, J. A., Jr., & Isenberg, I. (1974b) *Biochemistry 13*, 2098-2104.
Gurley, L. R., Tobey, R. A., Walters, R. A., Hildebrand, L. E., Hohmann, P. G., D'Anna, J. A., Barham, S. S., & Deaven, L. L. (1978) in *Cell Cycle Regulation* (Jeter, J. R., Jr., Cameron, I. L., Padilla, G. M., & Zimmermann, A. M., Eds.) pp 37-60, Academic Press, New York.
Johnson, E. M. (1977) *Adv. Cyclic Nucleotide Res. 8*, 267-309.
Jungmann, R. A., Lee, S., & DeAngelo, A. B. (1975) *Adv. Cyclic Nucleotide Res. 5*, 281-306.
Kobayashi, Y., & Iwai, K. (1970) *J. Biochem. (Tokyo) 67*, 465-472.
Kornberg, R. D. (1977) *Annu. Rev. Biochem. 46*, 931-954.
Kurochkin, S. N., Andreeva, N. B., Gasaryan, K. G., & Severin, E. S. (1977) *FEBS Lett. 76*, 112-114.
Laemmli, U. K. (1970) *Nature (London), New Biol. 227*, 680-685.
Ruiz-Carrillo, A., & Allfrey, V. G. (1973) *Arch. Biochem. Biophys. 154*, 185-191.
Schaeppi, Y., & Treadwell, W. D. (1948) *Helv. Chim. Acta 31*, 577-588.
Shoemaker, C. B., & Chalkley, R. (1978) *J. Biol. Chem. 253*, 5802-5807.
Yeaman, S. J., Cohen, P., Watson, D. L., & Dixon, G. H. (1977) *Biochem. J. 162*, 411-421.
Zick, Y., Cesla, R., & Shaltiel, S. (1979) *J. Biol. Chem. 254*, 879-887.

Ligand-Induced Transfer of Proteins between Phases: Dependence upon the Strength of Ion Pair Interactions†

Robert V. Mustacich‡ and Gregorio Weber*

ABSTRACT: Chemical modifications of ionizable groups of bovine serum albumin and lysozyme are described which lessen both pH and solvent polarity restrictions to the transfer of the proteins from water to alcohol phases on addition of suitable anions. Esterification of one-third of the carboxyl groups of albumin with triethyloxonium fluoroborate yielded protein fractions which could be transferred, by addition of *p*-toluenesulfonate or octylsulfonate, into butanol at pH 4.8 but not pH 7.1, while the intact protein could only be transferred at pH 2.4. Further, guanidation of 30% of the ϵ-amino groups yielded an ethylated-guanidated albumin which could be transferred into 1-butanol at pH 7.1 or into 1-octanol at pH 2.4. This notable increase in the ease of partition upon guanidation is directly traceable to the higher stability of the neutral ion pairs formed by guanidine ($pK \sim 13$) above those formed by the ϵ-amino groups of lysine ($pK \sim 10$). Differences in the partition of intact albumin and lysozyme at pH 2.4 are explained by their different arginine/lysine ratios. The rotational relaxation times of modified dansylated protein–ligand complexes in the alkanols were measured by combining fluorescence polarization, fluorescence lifetime, and alcohol viscosity data, over the range of 2–38 °C. The times observed (10–25 ns) are much shorter than those in water, indicating a large increase in the internal motions of the protein in the less polar solvent in the nanosecond range.

The ability to alter the partition equilibrium of a protein between an aqueous phase and an organic phase by the binding of ligands to the protein is a fundamental concept in understanding the interaction of proteins with membranes. Further, gating phenomena may achieve their sensitivity by combining ligand-induced partition changes of the receptor protein with electrophoresis in the presence of a transmembrane potential. Ligand-stabilized interfacial electrophoresis is a sensitive, on–off switchable function of ligand concentration even in the presence of electric fields weak relative to the electric fields of biological membranes. This has been demonstrated with serum albumin and lysozyme at pH 2.4 in a saturated water–butanol system with *p*-toluenesulfonate as the ligand (Mustacich & Weber, 1978). High reaction orders of ligand

† From the Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. *Received August 31, 1979.* This work was supported by Grant 11223 of the National Institute of General Medical Sciences, U.S. Public Health Service.

‡ Present address: The Procter and Gamble Co., Miami Valley Laboratories, Cincinnati, OH 45247.

0006-2960/80/0419-0990$01.00/0