peptides used in this work and Drs. Ph. Wahl, G. Laustriat, and D. Gérard for help in fluorescence lifetime measurements.

References

Backer, J. M., Molin, Y. N., and Sorokin, A. S. (1970), *Biochem. Biophys. Res. Commun. 38,* 871.
Brown, P. E. (1970), *Biochim. Biophys. Acta 213,* 282.
Daune, M. (1972), *Eur. J. Biochem. 26,* 207.
Dimicoli, J. L., and Hélène, C. (1971), *Biochimie 53,* 331.
Dimicoli, J. L., and Hélène, C. (1973), *J. Amer. Chem. Soc. 95,* 1036.
Dimicoli, J. L., and Hélène, C. (1974a), *Biochemistry 13,* 714.
Dimicoli, J. L., and Hélène, C. (1974b), *Biochemistry 13,* 724.
Durand, M., Maurizot, J. C., Borazan, H. N., and Hélène, C. (1975), following paper.
Feitelson, J. (1964), *J. Phys. Chem. 68,* 391.
Fritzsche, H. (1972), *FEBS Lett. 23,* 105.
Gabbay, E. J., Sanford, K., and Baxter, C. S (1972), *Biochemistry 11,* 3429.
Gabbay, E. J., Sanford, K., Baxter, C. S., and Kapicak, L. (1973), *Biochemistry 12,* 4021.
Hélène, C. (1971a), *Nature (London), New Biol. 234,* 120.
Hélène, C. (1971b), *FEBS Lett. 17,* 73.
Hélène, C. (1973), *Photochem. Photobiol. 18,* 255.
Hélène, C., and Dimicoli, J. L. (1972), *FEBS Lett. 26,* 6.
Hélène, C., Dimicoli, J. L., and Brun, F. (1971a), *Biochemistry 10,* 3802.
Hélène, C., Montenay-Garestier, Th., and Dimicoli, J. L. (1971b), *Biochim. Biophys. Acta 254,* 349.
McGhee, J. D., and Von Hippel, P. H. (1974), *J. Mol. Biol. 86,* 469.
Montenay-Garestier, Th. (1974), Conference on Excited States of Biological Molecules, Lisbon, 1974.
Montenay-Garestier, Th., and Hélène, C. (1968), *Nature (London) 217,* 844.
Montenay-Garestier, Th., and Hélène, C. (1971), *Biochemistry 10,* 300.
Novak, R. L., and Donhal, J. (1973), *Nature (London), New Biol. 243,* 155.
Parker, C. A. (1968), Photoluminescence of Solutions, Amsterdam, Elsevier.
Sander, C., and Ts'o, P. O. P. (1971), *J. Mol. Biol. 55,* 1.
Saxinger, C., Ponnamperuma, C., and Woese, C. (1971), *Nature (London), New Biol. 234,* 172.
Sellini, H., Maurizot, J. C., Dimicoli, J. L., and Hélène, C. (1973), *FEBS Lett. 30,* 219.
Toulmé, J. J., Charlier, M., and Hélène, C. (1974), *Proc. Nat. Acad. Sci. U.S. 71,* 3185.
Wagner, K. G., and Lawaczeck, R. (1972), *J. Magn. Reson. 8,* 164.
Wahl, Ph. (1969), *Biochim. Biophys. Acta 175,* 55.

Interaction of Aromatic Residues of Proteins with Nucleic Acids. Circular Dichroism Studies of the Binding of Oligopeptides to Poly(adenylic acid)†

Maurice Durand, Jean-Claude Maurizot, Hanna N. Borazan,‡ and Claude Hélène*

ABSTRACT: The binding of peptides containing lysyl and aromatic residues to poly(A) in its single-stranded form at pH 7 leads to a change of its circular dichroism (CD) spectrum, which is mainly due to the stacking of the aromatic amino acid with the bases of poly(A). Comparison is made between the binding of peptides having different primary structures which gives indications on the way the peptides bind to poly(A). A method is described which allows the calculation of the binding parameters from CD data. The magnitude of the association constant depends on the size of the aromatic ring and decreases in the order tryptophan > tyrosine > phenylalanine. The CD amplitude decreases linearly with the concentration of bound molecules. These results are discussed with respect to the role played by aromatic amino acids in complex formation between nucleic acids and proteins.

In previous papers we have shown that aromatic amines could interact with poly(A) to form stacked complexes with adenine bases (Hélène *et al.*, 1971a,b; Durand *et al.*, 1975). This leads to an unstacking of the adenine bases of poly(A) as shown by the change in the circular dichroism (CD) spectrum, as well as in the proton magnetic resonance (pmr) spectrum of poly(A) (Hélène *et al.*, 1971a,b; Razka and Mandel, 1971). This study has been extended to oligopeptides containing aromatic residues. Attention has been focused on peptides whose general formula is Lys-X-Lys, where X is tryptophan, tyrosine, phenylalanine, and, for comparison, alanine. At neutral pH, these peptides bear three positive and one negative charge, so that we can expect that the binding to poly(A) will be enhanced as compared to the aromatic amines as long as it depends on electrostatic forces. Furthermore, due to the increase of the binding constant, it has been possible to obtain a better

† From the Centre de Biophysique Moléculaire, CNRS, 45045 Orleans Cedex, France. *Received May 13, 1974.* This work has been supported by the Délégation Générale à la Recherche Scientifique et Technique (Contract No. 72-7-0498, ACC Interactions Moléculaire en Biologie).

‡ Present address: Department of Pharmaceutical Chemistry, Baghdad University, Baghdad, Iraq.

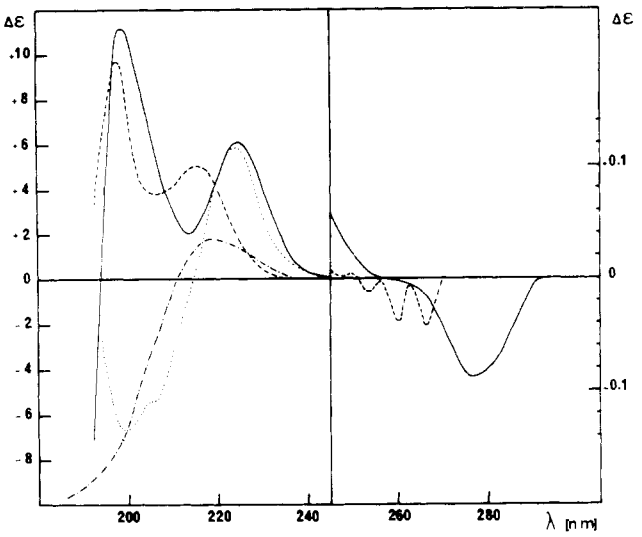

FIGURE 1: CD spectra of the peptides Lys-X-Lys at 1°, pH 7 (note that on the right part of the figure the scale is expanded 50-fold): (—) Lys-Tyr-Lys; (- - -) Lys-Phe-Lys; (• • •) Lys-Trp-Lys; (• - •) Lys-Ala-Lys.

quantitative analysis of the binding process. We have also studied the effect on the poly(A) CD spectrum of peptides having different primary structures (Lys-TyrNH_2, Ala-TyrNH_2, Tyr-Lys, etc.) in order to get a better understanding of the mechanism of interaction. Different physical methods have been used to investigate the binding of the oligopeptides to poly(A). We present here the results of our CD studies. Proton magnetic resonance results are published elsewhere (Hélène and Dimicoli, 1972; Dimicoli and Hélène, 1974a,b) and fluorescence results are presented in the accompanying paper (Brun *et al.*, 1975).

Experimental Methods

L-Lysyl-L-tyrosinamide (Lys-TyrNH_2), glycyl-L-tryptophan (Gly-Trp), L-tryptophanamide (TrpNH_2), L-tyrosinamide (TyrNH_2), L-tryptophanglycinamide (Trp-GlyNH_2), and L-alanyl-L-tyrosinamide (Ala-TyrNH_2) were purchased from Cyclo Chemical. L-Lysyl-L-tryptophyl-L-lysine (Lys-Trp-Lys), L-lysyl-L-tyrosyl-L-lysine (Lys-Tyr-Lys), L-tryptophylglycine (Trp-Gly), L-tryptophyl-L-lysine (Trp-Lys), L-tyrosylglycine (Tyr-Gly), glycyl-L-tyrosine (Gly-Tyr), and L-tyrosyl-L-lysine (Tyr-Lys) were purchased from Mann.

L-Lysyl-L-phenylalanyl-L-lysine (Lys-Phe-Lys), L-lysyl-L-alanyl-L-lysine (Lys-Ala-Lys), L-lysyl-L-*O*-methyltyrosyl-L-lysine (Lys-TyrOMe-Lys), L-lysyltryptophan methyl ester (Lys-TrpOMe), and L-lysyl-L-lysyl-L-tryptophan methyl ester (Lys-Lys-TrpOMe) were synthesized in our laboratory by Dr. J. Rossi.

Poly(A) and poly(C) were purchased from Miles. Solutions were made in a buffer containing NaCl (1 mM), sodium cacodylate (1 mM), and EDTA (0.2 mM) at pH 7. In order to adjust this low ionic strength, poly(A) was extensively dialyzed in the cold room against this buffer.

Circular dichroism (CD) spectra were recorded with a Roussel Jouan dicrograph in a thermostated quartz cell.

Results

Circular Dichroism of the Peptides. The investigated peptides have optical activity which may interfere with the CD of poly(A) in contrast to aromatic amines whose binding to poly(A) has been described elsewhere (Durand *et al.*, 1975). The CD spectra of the peptides Lys-X-Lys are shown in Figure 1. These CD spectra may be divided in two regions. At wavelengths longer than 240 nm, the CD due to the asymmetrically perturbed aromatic chromophore is small. For example, the amplitude of the Lys-Tyr-Lys CD spectrum at the wavelength of its maximum (275 nm) is 200 times smaller than that of poly(A). With Lys-Trp-Lys the signal is too weak to be detected with our CD instrument. At wavelengths below 240 nm the spectrum is due not only to the aromatic chromophores but also to the different amide transitions. In this region the amplitude is larger, and in some cases it may be of a magnitude similar to that of poly(A). The positions of the different CD bands observed agree very well with those of small peptides containing similar aromatic residues (for a review see Adler *et al.*, 1973).

In order to avoid difficulties due to the optical activity of the peptide we have limited our measurements to wavelengths longer than 240 nm. In this region we have subtracted (if it exists) the contribution of the peptide. It may be anticipated that this will be justified by the fact that the changes induced by peptide binding on the poly(A) CD spectrum are at least one order of magnitude larger than the CD of the peptide alone (see below). Detailed studies of the CD of the different peptides will be published separately.

Change in the CD Spectrum of Poly(A) Induced by Peptide Binding. Addition of the peptides Lys-X-Lys to poly(A) induces important changes in the CD spectrum of this polymer (Figure 2). These changes depend markedly on the nature of the central residue X. Lys-Ala-Lys induces a small decrease of the intensity of the positive band and a weak increase of that of the negative band. With peptides containing an aromatic residue (Lys-Trp-Lys, Lys-Tyr-Lys, and Lys-Phe-Lys) there is a large decrease of the amplitude of the two bands. However, the intensity decrease is larger for the positive than for the negative band. It should be noted that there is no marked shift in the wavelengths of the two maxima. These results differ slightly from those obtained with the aromatic amines (Durand *et al.*, 1975) which induced similar changes in both the positive and the negative bands. This is better illustrated in Figure 3 which shows the difference CD spectra between poly(A) alone and its equimolar mixture with the peptides. A quantitative analysis of CD variations will be presented below from which it can be deduced that in equimolar mixtures the extent of binding of the different peptides is nearly the same. With aromatic amines (illustrated in Figure 3 by tryptamine) difference spectra are similar in shape to that of poly(A). This is not the case with Lys-X-Lys when X is an aromatic residue. Particularly one can notice an increase in the ratio of the intensities of the positive to the negative band (Table I). With Lys-Ala-Lys the difference spectrum is composed of only one broad band of weak intensity. From the comparison between these spectra one can assume that the difference spectrum due to Lys-X-Lys with X aromatic is composed of two contributions: one similar to that obtained with Lys-Ala-Lys and one similar in shape to the CD spectrum of poly(A). As shown in Figure 3 the first contribution must be small.

It is clear that the change in the poly(A) CD spectrum is not due to a circular dichroism induced in the peptide absorption bands. This would be possible only for peptides having a chromophore absorbing between 240 and 300 nm. The similarity of the CD difference spectra for Lys-Tyr-

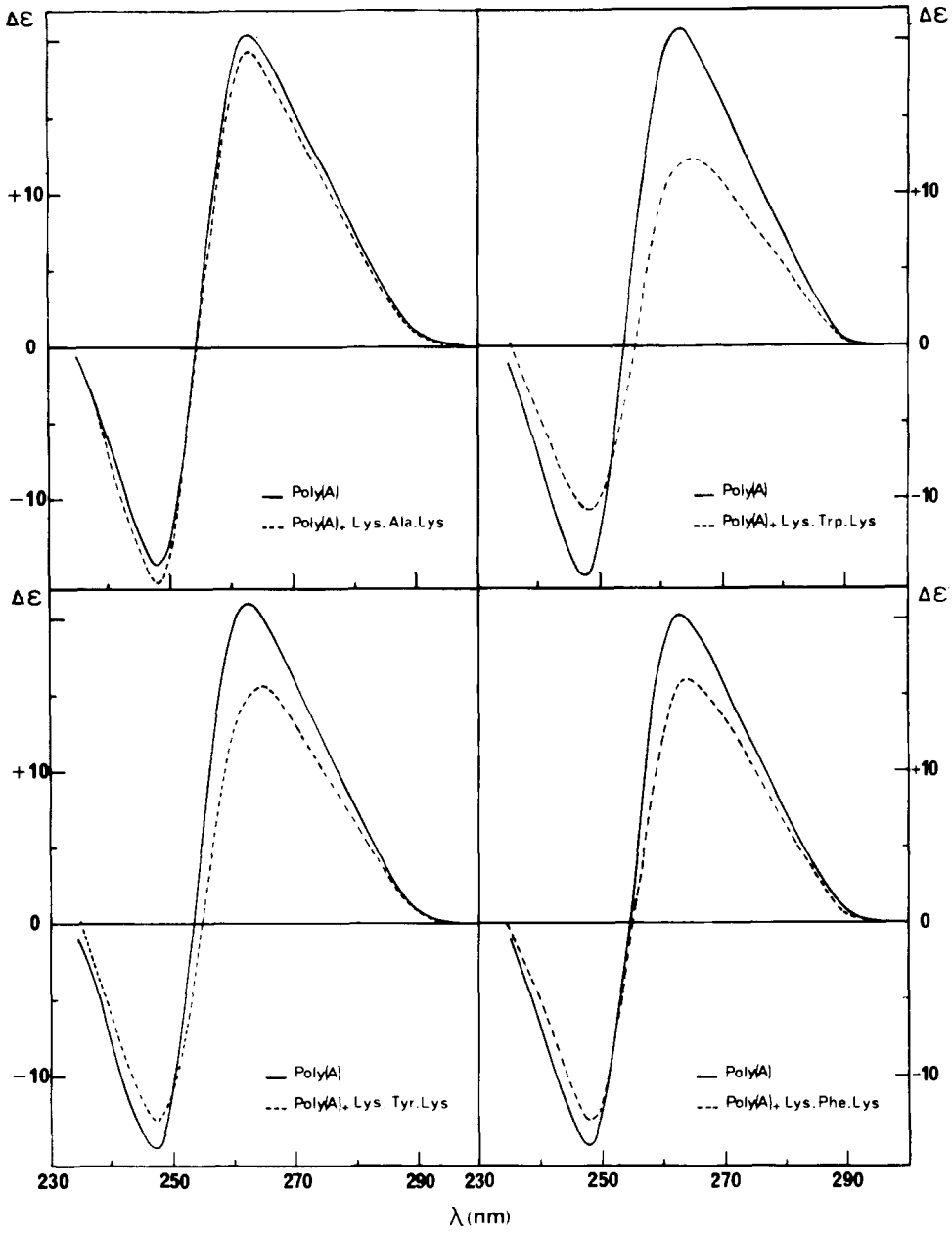

FIGURE 2: CD spectra of 5×10^{-4} M poly(A) in the absence (—) and the presence (- - -) of equimolar concentrations of peptides Lys-X-Lys at pH 7 (10^{-3} M sodium cacodylate-10^{-3} M NaCl-2×10^{-4} M EDTA), temperature 1°.

Lys, Lys-Phe-Lys, and Lys-Trp-Lys, whose absorption spectra greatly differ from one another, excludes induced dichroism in the aromatic amino acid absorption bands as a major contribution to the change in the CD spectrum. For the same reason, we can exclude a conformational change of the peptide as an important contribution to the CD difference spectra.

One can conclude that the changes observed in the CD spectrum correspond to a conformational change induced in poly(A) by peptide binding. In a previous paper (Durand *et al.*, 1975), we have attributed this conformational change to the unstacking of poly(A) bases induced by base stacking with the aromatic ring of the amines. Such an unstacking is probably induced by the binding of the aromatic peptides as well, but there is a second contribution to the conformational change which is due to electrostatic binding of the lysyl residues as shown in the case of Lys-Ala-Lys which gives only this second type of interaction.

At pH 7, peptides Lys-X-Lys bear three positive charges (terminal NH_3^+ and two lysyl ϵ-NH_3^+) and one negative charge (terminal COO^-). The N-terminal lysyl residue is similar to a diamine. Several investigations have shown that diamines interact with polynucleotides (Higushi and Tsuboi, 1965; Szer, 1966; Rogers *et al.*, 1967; Gabbay and Shimshack, 1968; Glaser and Gabbay, 1968). One possibility is that the conformational change induced by Lys-Ala-Lys is only due to the binding of the N-terminal lysyl residue to the polynucleotide (as will be shown later, the second lysine residue is not involved in the binding of the peptide to poly(A)). In order to test this hypothesis we investigated the change of the CD spectrum of poly(A) in the presence of cadaverine (1,5-diaminopentane, $NH_3^+(CH_2)_5NH_3^+$). In this compound the two NH_3^+ groups are separated by the same number of methylene groups as in a lysyl residue. In an equimolar mixture of this compound with poly(A) a small decrease of the intensity of the positive band (about

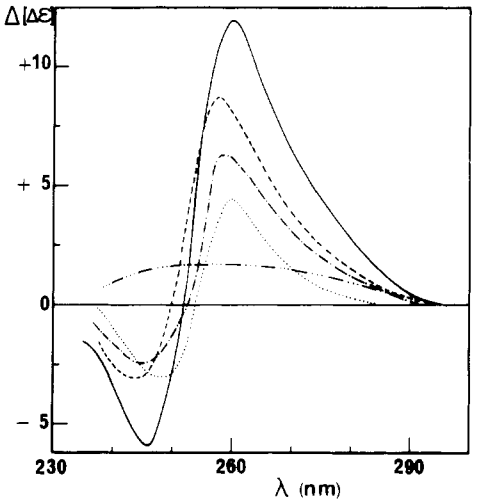

FIGURE 3: CD spectra difference between poly(A) alone and in the presence of equimolar concentration of peptides. Same condition as for Figure 2: (—) Lys-Trp-Lys; (- - -) Lys-Tyr-Lys; (· - - - ·) Lys-Phe-Lys; (- · · · -) Lys-Ala-Lys; (· · ·) tryptamine.

Table I: Characteristics of the Circular Dichroism Difference Spectra between Poly(A) and Poly(A)–Peptide Complexes.[a]

	Poly(A)[b]	Poly(A)–Lys-Trp-Lys	Poly(A)–Lys-Tyr-Lys	Poly(A)–Lys-Phe-Lys	Poly(A)–5-Methoxytryptamine
λ_1	262.5	260	260	259	261
λ_2	247.5	245.5	246.5	245	247.5
R	1.43	2	2.66	2.45	1.5

[a] λ_1 and λ_2 are the wavelengths of the maximum of the positive and negative bands in the difference spectrum, respectively. R is the ratio between the intensities of the positive and negative bands in the difference spectrum. [b] Values are from the CD spectrum of poly(A) alone.

5%) is observed whereas the negative band is not affected. Addition of lysine methyl ester (LysOMe) induces a similar change of the CD spectrum of poly(A). These modifications are very similar to those induced by Lys-Ala-Lys and may be interpreted as the result of electrostatic binding of these molecules to poly(A). Such a binding, involving probably two adjacent phosphate groups, may induce small conformational changes (*e.g.*, tilting or rotation of two adjacent bases with respect to each other) and give rise to the observed modification of the CD spectrum of poly(A). The binding of poly(L-lysine) to poly(A) induces a very large change in its optical rotatory dispersion (ORD) spectrum (Davidson and Fasman, 1969). Such a large change is not observed when Lys-Ala-Lys binds to poly(A) (Figure 2).

From the comparison between the changes in CD spectra induced by the binding of Lys-Ala-Lys and Lys-X-Lys (X aromatic) it may be concluded that the main effect on the CD spectrum of poly(A) can be attributed to the aromatic ring which induces unstacking of the adenine rings. Fluorescence and pmr experiments provide evidence for a stacking between adenine bases and the aromatic residue of Lys-X-Lys. Such a stacking induces an unstacking of poly(A) bases (Dimicoli and Hélène, 1974a,b) in agreement with the CD results.

In all cases, addition of NaCl reverses the observed decrease of the CD spectrum of poly(A), demonstrating the importance of electrostatic interactions in peptide binding. It has also been shown by other techniques (fluorescence and nuclear magnetic resonance (nmr)) that increasing the ionic strength leads to complex dissociation.

Using the changes of the CD intensity at a given wavelength it is possible to follow the binding of the peptide to poly(A). Figure 4 illustrates these binding studies and shows, for comparison, the curves obtained with tryptamine. As the concentration of peptide is increased, the relative change of the CD amplitude seems to tend toward a limit, but this limit is not reached in the concentration range allowed by the absorption of the peptide. As reported elsewhere for aromatic amines (Durand *et al.*, 1975) the size of the ring is an important factor in determining the magnitude of the observed decrease.

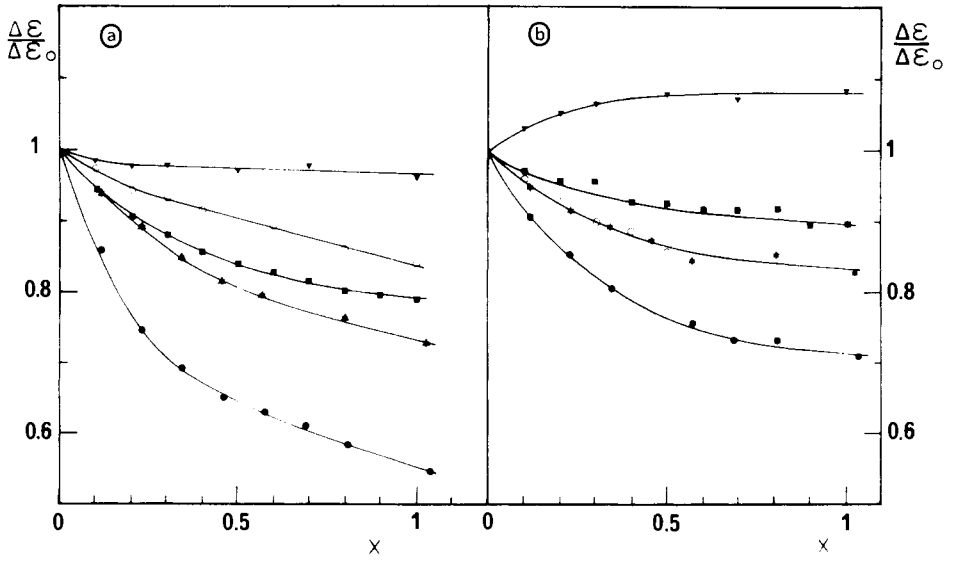

FIGURE 4: Concentration dependence of the intensity of the CD signal of poly(A) for various peptides. Same conditions as in Figure 2: (▼) Lys-Ala-Lys; (●) Lys-Trp-Lys; (★) Lys-Tyr-Lys; (■) Lys-Phe-Lys; (○) tryptamine. X is the ratio of peptide and poly(A) concentrations. The concentration of poly(A) is constant and equal to 5×10^{-4} M. The change in CD amplitude is followed at 262.5 nm (a) and at 247.5 nm (b).

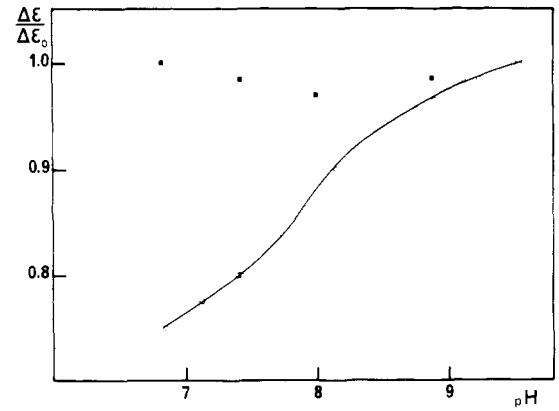

FIGURE 5: pH dependence of $\Delta\epsilon/\Delta\epsilon_0$, the relative decrease of the CD signal, of poly(A) at 262.5 nm alone (3.9×10^{-4} M) (■) and in the presence of Lys-Trp-Lys (4.25×10^{-4} M) (○).

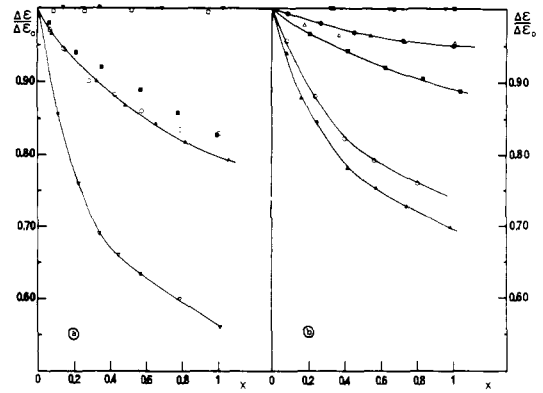

FIGURE 6: Variation of the intensity of the CD signal of poly(A) at 262.5 nm, for peptides of different sequences; same conditions as in Figure 2: (a) (▼) Gly-Trp, (□) Trp-Gly, (■) $TrpNH_2$, (○) Trp-Lys, (▲) $Trp\text{-}GlyNH_2$, (▽) Lys-Trp-Lys; (b) (▼) Tyr-Gly, (□) Gly-Tyr, (●) $Ala\text{-}TyrNH_2$, (△) $TyrNH_2$, (■) Tyr-Lys, (○) $Lys\text{-}TyrNH_2$, (▲) Lys-Tyr-Lys.

Influence of pH. The terminal NH_3^+ group of the investigated oligopeptides is involved in the binding process as shown by the pH dependence of complex formation. This particular NH_3^+ group has a pK value around 8 as determined by fluorescence (Brun *et al.*, 1975), nmr (Dimicoli and Hélène, 1974a,b), and CD measurements. Titration of an equimolar mixture of poly(A) and Lys-Trp-Lys shows an increase of the intensity of the CD spectrum when the pH increases until the value of poly(A) alone is reached (Figure 5). This clearly demonstrates that binding of Lys-Trp-Lys to poly(A) is greatly reduced when the N-terminal group is unprotonated. Similar conclusions were obtained from the pH dependence of the fluorescence and nmr spectra (Brun *et al.*, 1975; Hélène *et al.*, 1973; Dimicoli and Hélène, 1974a,b).

Influence of the Primary Structure of the Peptide. Various peptides containing tryptophan or tyrosine residues with other different amino acids were studied in order to determine how the sequence and the nature of these adjacent residues interfere with the interaction of the phenol or indole ring with the bases of poly(A). We studied the binding to poly(A) of six peptides containing a tryptophyl residue (Gly-Trp, Trp-Gly, Trp-Lys, $TrpNH_2$, $Trp\text{-}GlyNH_2$, and Lys-Trp-Lys) and of seven peptides containing a tyrosyl residue (Tyr-Gly, Gly-Tyr, $TyrNH_2$, $Ala\text{-}TyrNH_2$, Tyr-Lys, $Lys\text{-}TyrNH_2$, and Lys-Tyr-Lys). As shown in Figure 6, the behavior of the poly(A)–peptide systems is correlated with the total charge of the peptide. Among the tryptophan-containing peptides there are clearly three groups of compounds. Gly-Trp and Trp-Gly bear one positive (the NH_3^+ terminal) and one negative (the COO^- terminal) charge. These compounds induce practically no change in the CD spectrum of poly(A). The second group of peptides has a total positive charge of one. This may be the result of either two positive and one negative charge (Trp-Lys) or of only one positive charge ($TrpNH_2$, $Trp\text{-}GlyNH_2$). Lys-Trp-Lys, which has three positive and one negative charge, gives rise to a more pronounced effect. We also tried Lys-Lys-TrpOMe which bears three positive charges. Under our experimental conditions addition of this peptide to poly(A) leads to a precipitation when the concentration ratio increases beyond 0.2. Such a phenomenon does correspond to a strong binding to poly(A). The same conclusions concerning the influence of the total charge of the peptides may also be reached from a comparison between peptides containing tyrosine residues (Figure 6). Within each group of peptides containing the same aromatic amino acid and corresponding to the same total charge there are small differences in the effect upon the CD spectrum of poly(A). This may reflect modifications in the charge distribution in the peptide structure which may induce changes in the binding constant to poly(A). This may also result from the fact that different conformations of the peptides do not allow the same overlap of the aromatic ring with adenine bases. These possibilities are not exclusive and the observed variation may result from these two contributions.

Figure 6 shows that the binding curve of $Lys\text{-}Tyr\text{-}NH_2$ is very similar to that of Lys-Tyr-Lys, which might indicate that the C-terminal lysine residue is not involved in complex formation with poly(A).

Quantitative Analysis of CD Data. When a ligand binds to a polymer to form only one type of complex, the same relative change of the CD amplitude of the polymer ($\Delta\epsilon/\Delta\epsilon_0$) corresponds to the same degree of saturation. The ratio of ligand and polymer concentrations that will give the same value of $\Delta\epsilon/\Delta\epsilon_0$ will depend on polymer concentration (P_0) according to eq 1, where r is the degree of saturation of

$$X = r + C_f/P_0 \qquad (1)$$

the polymer ($r = C_b/P_0$), C_f and C_b being the concentrations of free and bound ligand, respectively.

If several complexes exist in equilibrium, and if the distribution of bound molecules between the different types of complexes does not depend on polymer concentration (*i.e.*, either cooperativity is not involved for any of the complexes or, if cooperativity is involved, the same cooperativity parameter applies to all types of complexes), eq 1 can still be used with $r = \sum_i r_i$ even if one (or more) complex does not contribute to the change in the CD spectrum.

The relative decrease of the amplitude of the poly(A) CD spectrum depends on the concentration of both poly(A) and peptide. We have shown in the accompanying paper (Brun *et al.*, 1975) that the binding between poly(A) and peptides involves the formation of two types of complexes:

$$\text{poly(A)} + \text{free peptide} \underset{K_1}{\rightleftharpoons} \text{complex A} \underset{K_2}{\rightleftharpoons} \text{complex B}$$

The above analysis (eq 1) may be applied to such an equilibrium. Figure 7 shows that plots of x *vs.* $1/P_0$ give straight lines as predicted from eq 1. Values of r and C_f were obtained from such plots. A Scatchard representation was then used as shown in Figure 7. Our method of calculation of r and C_f will give us a value of r corresponding to the sum

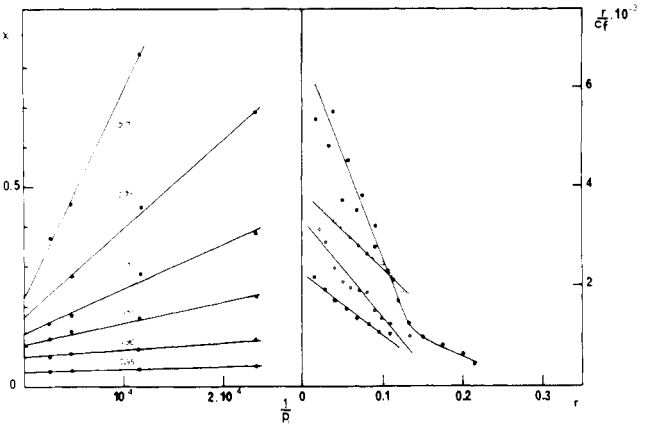

FIGURE 7: Calclulation of the binding parameters for the association of peptides with poly(A). (Left) Plots of X *vs.* $1/P_0$ according to eq 1. Numbers indicated on the lines correspond to the same relative decrease ($\Delta\epsilon/\Delta\epsilon_0$) of the CD amplitude of poly(A) at different concentrations. X is the ratio of peptide to poly(A) concentration and P_0 is the concentration of poly(A). (Right) Scatchard analysis for the binding of the peptides Lys-X-Lys to poly(A): (●) Lys-Trp-Lys; (☆) Lys-Tyr-Lys; (□) Lys-TyrOMe-Lys; (■) Lys-Phe-Lys.

Table II: Binding Parameters for the Association of Peptides to Poly(A).

Peptide	T (°C)	$10^{-4}K_{app}$ (M^{-1})	a^a
Lys-Trp-Lys	2.5	6.6	1.47
	19.8	5.0	1.47
Lys-Tyr-Lys	2.5	3.3	1
Lys-Tyr OMe-Lys	2	3.9	1
Lys-Phe-Lys	2.5	2.2	0.73

[a] a is the slope of the plots of $\Delta\epsilon/\Delta\epsilon_0$ *vs.* r (see eq 2).

of the two complexes A and B. Consequently an apparent association constant (K_{app}) will be calculated from CD data which is related to K_1 and K_2 by the relationship $K_{app} = K_1(1 + K_2)$.

At high values of r, Scatchard plots show a curvature (see the case of Lys-Trp-Lys in Figure 7). As discussed in the preceding paper, dealing with fluorescence measurements, this behavior is expected for the binding of a ligand which covers more than one phosphate group (McGhee and Von Hippel, 1974). The y-axis intercept corresponds to the value of the apparent binding constant K_{app}, whereas the intercept of the approximately linear initial part of the curve with the x axis is related to the number n of residues covered by the peptide by the relationship $r_{intercept} = 1/(2n - 1)$. This analysis gave us values of n between 3 and 4 for the different peptides. A peptide bound to poly(A) will thus cover three to four phosphate groups. It should be noted that anticooperativity may also contribute to the curvature of the Scatchard plots, as it can be expected that the binding constant decreases as binding proceeds due to a decrease of the electrostatic potential of the polynucleotide and to electrostatic repulsion between bound peptides. Values of the apparent binding constants are given in Table II. These values, as well as the values of n, are in good agreement with those obtained from fluorescence data (Brun *et al.*, 1975).

As already observed in the case of aromatic amines (Durand *et al.* 1975), the CD amplitude of poly(A) decreases linearly as a function of r (in the range of r values investigated) according to eq 2 (see Figure 8).

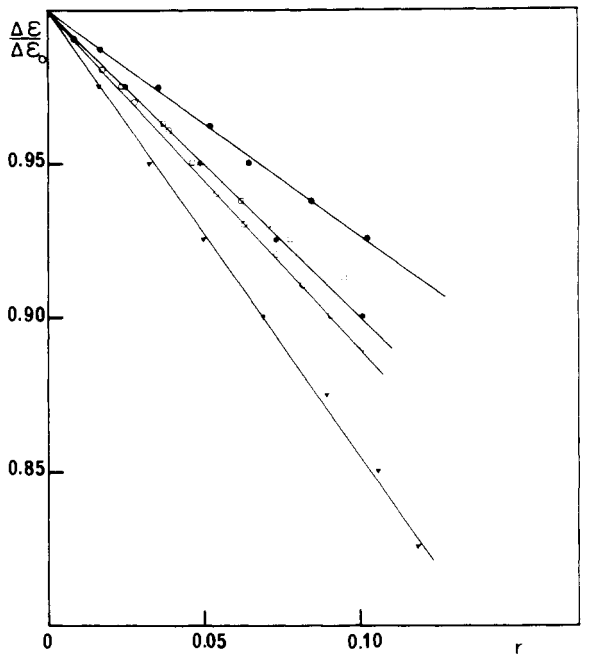

FIGURE 8: Relative decrease of the CD amplitude of poly(A) at 262.5 nm ($\Delta\epsilon/\Delta\epsilon_0$) as a function of the degree of saturation (r) of the polynucleotide: (○) 5-methoxytryptamine; (★) Lys-TyrOMe-Lys; (□) Lys-Tyr-Lys; (▼) Lys-Trp-Lys; (●) Lys-Phe-Lys.

$$\Delta\epsilon/\Delta\epsilon_0 = 1 - ar \quad (2)$$

If several types of complexes are formed and if they give additive contributions to the conformational changes, eq 2 becomes:

$$\frac{\Delta\epsilon}{\Delta\epsilon_0} = 1 - \sum_i a_i r_i \quad (3)$$

The slopes of the straight lines obtained by plotting $\Delta\epsilon/\Delta\epsilon_0$ *vs.* r are given in Table II. These values can be thought of as representing the average number of adenine bases whose contribution to the CD spectrum of poly(A) has been eliminated per bound peptide. The total effect per bound Lys-X-Lys peptide decreases in the order Trp > Tyr > Phe. This order is in agreement with that found with aromatic amines (Durand *et al.*, 1975).

Discussion and Conclusion

Oligopeptides Lys-X-Lys bind to poly(A) by electrostatic interactions. This is demonstrated by the effect of the ionic strength and by pH-titration experiments. As shown by the comparison between different peptides and by the titration experiments, the N-terminal lysyl residue is involved and is probably bound to two adjacent phosphates. This binding poorly affects the conformation of poly(A) as shown by the small modification of the CD spectrum induced by the binding of Lys-Ala-Lys or cadaverine. When X is an aromatic residue a second conformational change is superimposed due to the stacking of the aromatic amino acid with adenine bases. Such a stacking does not give rise to a detectable induced CD. Such an induced CD should exist, but it is difficult to predict its magnitude, which depends on the orientation and on the magnitude of the different dipole transition moments. It should be noted that nucleotides cov-

alently linked to aromatic amino acids (Gromova *et al.*, 1971) were found to give rise to very small induced optical activity.

Quantitative analysis of fluorescence data (quantum yields and lifetimes) has led us to postulate the formation of two types of complexes in equilibrium. In the first type (complex A), the peptide Lys-X-Lys is electrostatically bound to poly(A) but its aromatic ring does not interact with adenine bases; its fluorescence quantum yield and its pmr spectrum are identical with that of the free peptide. This complex should induce only small changes in the CD spectrum of poly(A). This type of complex is the only one formed when Lys-Ala-Lys binds to poly(A) and we have seen above that this binding leads to small conformational changes. In the second type of complexes (complex B), the peptide Lys-X-Lys is electrostatically bound to poly(A) and its aromatic ring interacts with bases. As shown in previous publications (Montenay-Garestier and Hélène, 1968, 1971; Hélène *et al.*, 1971b) stacking of aromatic amino acids with nucleic acid bases leads to a quenching of the amino acid fluorescence. The pmr spectrum of the amino acid is shifted toward higher fields (Hélène and Dimicoli, 1972; Dimicoli and Hélène, 1974a,b). Such a stacking interaction between adenine and aromatic amino acids should induce a destacking of neighboring adenine bases which leads to a conformational change manifested by a decrease of the CD amplitude of poly(A). This destacking of adenine bases is also observed by pmr measurements (Dimicoli and Hélène, 1974a,b).

There is a good agreement between the values of the binding parameters obtained by CD (Table II) and by fluorescence measurements (Tables I and II in the preceding paper). Circular dichroism results reflect the conformational changes of the polynucleotide induced upon peptide binding whereas fluorescence experiments are related to the behavior of the peptide itself. The quantitative agreement between these two methods which are concerned with two different physical aspects of the same interaction problem is a convincing argument of the validity of the model involving two types of complexes. The more likely mechanism of peptide binding to poly(A) is composed of two steps. In the first step the peptide binds to poly(A) to form an electrostatic complex. In the second step, there is a conversion of this external complex into a "stacked" complex. Molecular model building indicates that stacking of the aromatic amino acid with adenine bases can be done in two ways: an unwinding of the poly(A) with an increase of the interbase distance or a bending of the phosphate–sugar backbone. These two hypotheses lead to an unstacking of the adenine bases but the second one seems more likely because bending could affect more than one nucleotide residue as experimentally observed (Figure 8).

Quantitative analysis of CD data demonstrates that the apparent binding constant for poly(A) is larger for Lys-Trp-Lys than for Lys-Tyr-Lys. This clearly demonstrates that the binding is not exclusively electrostatic, as there is no difference of charge between these two peptides. Furthermore, the binding of one molecule of Lys-Trp-Lys induces larger perturbation in the CD spectrum of poly(A) than does Lys-Tyr-Lys (Figure 8). The association constant K_2 which represents the ratio of stacked and unstacked complexes is smaller for Lys-Trp-Lys than for Lys-Tyr-Lys (Brun *et al.*, 1975). Thus, the effect of bound peptide molecules on the CD spectrum of poly(A) is not related to the probability of stacking. This effect may be related to the size of the aromatic ring, the indole ring allowing better overlap with adenine.

Our CD investigations do not show qualitative differences in the mode of binding to poly(A) of peptides containing either tyrosine or tryptophan. It was previously shown in our laboratory that phenol derivatives could form hydrogen-bonded complexes with adenine (Sellini *et al.*, 1973), and we have suggested that the binding of tyrosine derivatives to DNA involves hydrogen bond formation (Dimicoli and Hélène, 1974b). Comparison of the CD changes induced in poly(A) by the binding of Lys-Tyr-Lys and of Lys-TyrOMe-Lys does not show any difference, ruling out the contribution of hydrogen-bonded complexes for Lys-Tyr-Lys. Thus, a single-stranded polynucleotide does not seem to differentiate between phenol and indole derivatives as does DNA.

The conformational changes of the nucleic acid induced by the interaction of an aromatic amino acid could be important in the formation of protein–nucleic acid complexes. A change in the nucleic acid conformation could be required for further biological activity of the complex to take place.

Preliminary experiments have shown that the CD spectrum of poly(C) is only slightly affected by the addition of the different peptides used in this study. Further studies on the nature of interactions between peptides and polynucleotide with different base composition should help understand the origin of the specific recognition of nucleic acids by proteins.

Acknowledgments

We wish to thank Dr. J. Rossi for the synthesis of some of the peptides used in this work.

References

Adler, A. J., Greenfield, N. J., and Fasman, G. D. (1973), *Methods Enzymol. 27,* 675.

Blake, A., and Peacocke, A. R. (1968), *Biopolymers 6,* 1225.

Brun, F., Toulmé, J. J., and Hélène, C. (1975), preceding paper.

Davidson, B., and Fasman, G. D. (1969), *Biochemistry 8,* 4116.

Dimicoli, J. L., and Hélène, C. (1974a), *Biochemistry 13,* 714.

Dimicoli, J. J., and Hélène, C. (1974b), *Biochemistry 13,* 724.

Dourlent, M., and Hélène, C. (1971), *Eur. J. Biochem. 23,* 86.

Durand, M., Borazan, H. N., Maurizot, J. C., Dimicoli, J. L., and Hélène, C. (1975), submitted for publication.

Gabbay, E. J., and Shimshack, R. R. (1968), *Biopolymers 6,* 255.

Glaser, R., and Gabbay, E. J. (1968), *Biopolymers 6,* 243.

Gromova, E. S., Tyaglov, B. V., and Zharabova, Z. A. (1971), *Biochim. Biophys. Acta 240,* 1.

Hélène, C., and Dimicoli, J. L. (1972), *FEBS Lett., 26,* 6.

Hélène, C., Dimicoli, J. L., Borazan, H. N., Durand, M., Maurizot, J. C., and Toulmé, J. J. (1973), *Jerusalem Symp. Quantum Chem. Biochem. 5,* 361.

Hélène, C., Dimicoli, J. L., and Brun, F. (1971a), *Biochemistry 10,* 3802.

Hélène, C., Montenay-Garestier, T., and Dimicoli, J. L.

(1971b), *Biochim. Biophys. Acta 254,* 349.
Higushi, S., and Tsuboi, M. (1965), *Bull. Chem. Soc. Jap. 39,* 1886.
McGhee, J. D., and Von Hippel, P. H. (1974), *J. Mol. Biol. 86,* 469.
Montenay-Garestier, T., and Hélène, C. (1968), *Nature (London) 217,* 844.
Montenay-Garestier, T., and Hélène, C. (1971), *Biochemistry 10,* 300.
Razka, M., and Mandel, M. (1971), *Proc. Nat. Acad. Sci. U.S. 68,* 1190.
Rogers, G. T., Ulbricht, T. L. V., and Szer, W. (1967), *Biochem. Biophys. Res. Commun. 27,* 372.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Sellini, H., Maurizot, J. C., Dimicoli, J. L., and Hélène, C. (1973), *FEBS Lett. 30,* 219.
Szer, W. (1966), *Biochem. Biophys. Res. Commun. 22,* 559.

The Formation of Tritiated *O*-Alkyl Lipid from Acyldihydroxyacetone Phosphate in the Presence of Tritiated Water†

Samuel J. Friedberg* and Aaron Heifetz

ABSTRACT: Previous studies from this laboratory on the mechanism of *O*-alkyl bond formation using a microsomal system from *Tetrahymena pyriformis* have shown that *O*-alkyl lipid synthesized from dihydroxyacetone phosphate has exchanged one hydrogen stereospecifically from the 1-sn position of the glycerol moiety. Indirect evidence suggested that acyldihydroxyacetone phosphate, an intermediate in *O*-alkyl lipid synthesis, is probably not the locus of the exchange. In the present study it was shown that stable acyldihydroxyacetone phosphate incubated in the presence of tritiated water and *Tetrahymena* microsomes does not become tritiated. When hexadecanol is added to the system *O*-alkyl lipid is produced which has incorporated one atom of hydrogen for each mole of hexadecanol at all time periods examined. Experiments in Ehrlich ascites tumor cells have shown that the hydrogen exchange also occurs in a mammalian system. The results indicate that the mechanism of *O*-alkyl lipid ether bond formation involves a hydrogen exchange and that this exchange occurs after the formation of acyldihydroxyacetone phosphate.

The reaction sequence leading to the formation of alkyl glycerolipids involves the formation of 1-acyldihydroxyacetone phosphate and replacement of the fatty acid by a long chain fatty alcohol with the formation of *O*-alkyldihydroxyacetone phosphate (Hajra, 1970; Wykle *et al.,* 1972). The keto group is reduced with NADPH followed by acylation. For further details on the biochemistry of ether lipids, the reader is referred to a recent extensive review (Snyder, 1972).

We have previously shown that when dihydroxyacetone phosphate (DHAP[1]) is used in the enzymatic formation of *O*-alkyl lipids, there is a hydrogen exchange from the carbon that acquires the *O*-alkyl moiety (Friedberg *et al.,* 1971; Friedberg and Heifetz, 1973). This exchange is specific for the same hydrogen labilized in the triosephosphate isomerase reaction (Friedberg *et al.,* 1972). The exchange was demonstrated through the loss of one tritium from [1,3-$^3H_2$]DHAP and by the acquisition of one tritium atom by *O*-alkyl lipids formed from DHAP in the presence of tritiated water. In the microsomal system from *Tetrahymena pyriformis,* a concomitant reaction is the coenzyme A dependent formation of dihydroxyacetone from dihydroxyacetone phosphate (Friedberg and Heifetz, 1973). The dihydroxyacetone formed also appears to have undergone a hydrogen exchange, and the coenzyme A requirement was interpreted to indicate that acyldihydroxyacetone phosphate (acyl-DHAP) is a precursor of dihydroxyacetone. In our earlier studies using the *Tetrahymena* system we were not able to isolate labeled acyl-DHAP from incubations which utilized [1,3-$^3H_2$]DHAP. We were, however, able to isolate acyldihydroxyacetone (acyl-DHA) which had not lost tritium. Thus the results suggested that the tritium exchange occurs after the formation of acyl-DHAP. However, we and others (Schroepfer and Bloch, 1965; Friedberg and Greene, 1967, Wood *et al.,* 1970) have encountered unexplained relative tritium enrichment in reactions which utilized mixtures of a substrate labeled with both ^{14}C and ^3H. Thus an actual tritium loss from acyl-DHA could have been masked by an artifactual enrichment or by a mechanism involving an isotope effect. We also considered that acyl-DHAP might have undergone a hydrogen exchange *via* an independent enzyme reaction similar to an isomerase or aldolase reaction. This might have accounted for the production of tritiated glyceryl ethers in our previous studies. Proof that a hydrogen exchange occurs at some point in an enzymatic reaction between acyl-DHAP and fatty alcohol is critical in discovering the mechanism of *O*-alkyl lipid

† From the Department of Medicine, The University of Texas Health Science Center at San Antonio, Texas. *Received May 23, 1974.* This work was supported by state appropriated research funds.

* Address all correspondence to this author at the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710.

[1] Abbreviations used are: DHAP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; acyl-DHA, acyldihydroxyacetone; acyl-DHAP, acyldihydroxyacetone phosphate; CAP, Hydroxy-3-chloro-2-propanone phosphate.