no exact, well-defined shape of the space constituting the binding site of the lipoprotein. It varies, taking the final shape that is compatible with the geometry of the retinal analogue. We believe that the view that the binding site has a large static empty space sufficient to accommodate all the different retinal analogues (further imposing some longitudinal restrictions) is not consistent with the experimental results reported here. For example, it cannot explain the varying degree of stability of the rhodopsin analogue in hydroxylamine, nor can it account for the failure to reverse the photoisomerization process from rhodopsin and 9-*cis*-rhodopsin back to 7-*cis*-rhodopsin.

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Effect of Hydrogen Bonding on Electronic Spectra and Reactivity of Flavins†

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ABSTRACT: Riboflavin tetrabutyrate undergoes characteristic spectral changes, in both the first and second absorption band regions, upon hydrogen bonding with trichloroacetic acid or trifluoroacetic acid. On the basis of the calculated electron densities, hydrogen bonding at the heteroatoms of the isoalloxazine nucleus is considered to occur with increasing concentrations of the proton donor, first at N(1), then at O(12), O(14), and N(3)H, and finally at N(5). The idea that the major effect of the hydrogen bonding at the N(1), N(3)H, and oxygen atoms of the flavin nucleus is to facilitate the electrophilicity of the N(5) position, which was predicted by molecular orbital calculations, was supported by the observation that the hydrogen-bonded flavin in its triplet state abstracts hydrogen from the donor *N*-benzyl-*N*,*N*′-dimethylethylenediamine at a faster rate than do the non-hydrogen-bonded species in CCl_4. The implications of the present study in the spectroscopic and catalytic properties of flavoproteins are briefly discussed.

The effect of hydrogen bonding on the electronic spectra of flavins has been studied by Yagi & Matsuoka (1956) and Kotaki et al. (1970). The effect of hydrogen bonding on the electronic structure and spectra of the flavin nucleus has also been described in terms of the self-consistent field molecular orbital method (Nishimoto et al., 1978). These studies suggested that the spectral characteristics of the flavin chromophore in *Clostridium* MP and *Desulfovibrio vulgaris* flavodoxins (Mayhew & Ludwig, 1975) can be at least partly accounted for in terms of hydrogen bonding, the occurrence of which in these proteins was verified by X-ray crystallography (Ludwig et al., 1976; Watenpaugh et al., 1976).

In the present work, we performed an experimental study using model systems in order to ascertain the possible consequence of hydrogen bonding for the spectral characteristics and catalytic reactivity of flavins. For the assessment of the catalytic reactivity of the flavin nucleus as an acceptor of electrons and nucleophiles, we adopted the flavin photoreactivity on the basis of our analysis of the flavin's N(5) elec-

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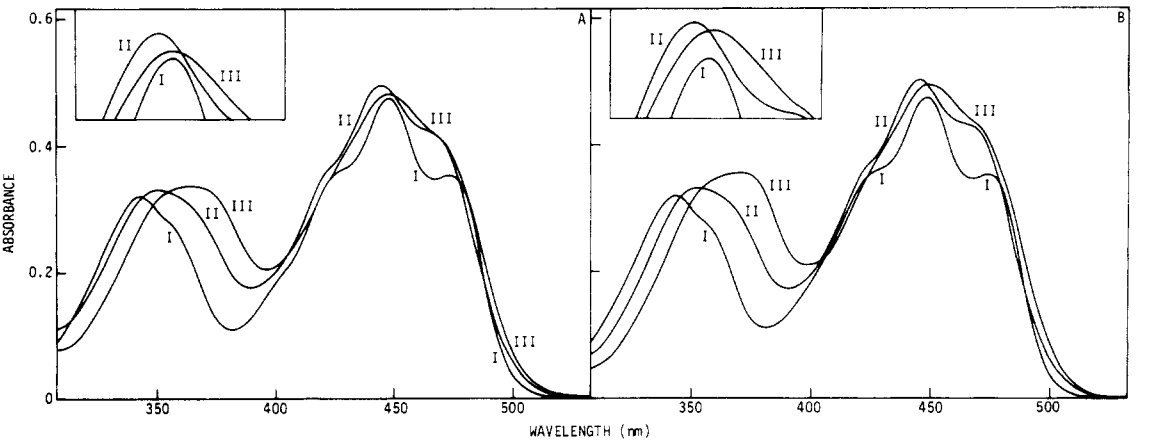


FIGURE 1: Effect of hydrogen bonding on the absorption spectrum of RFTB in CCl_4. Concentration of RFTB: 4×10^{-5} M. Concentrations of Cl_3AcOH or F_3AcOH: 0 (I); 1×10^{-3} (II); 1×10^{-2} M (III). (A) Cl_3AcOH; (B) F_3AcOH. The inserts are the enlarged figures around the first absorption band, and the curve numbers correspond to those of the original.

trophilicity, which is controlled by the frontier orbital (Sun & Song, 1973; Song & Sun, 1974). The N(5) reactivity of flavins has recently been reviewed by Bruice (1976a,b), Song et al. (1976), and Hemmerich (1976), among others (Massey et al., 1976; Maycock et al., 1976; Porter & Bright, 1976; Schonbrunn et al., 1976).

Materials and Methods

Materials. Riboflavin 2′,3′,4′,5′-tetrabutyrate (RFTB)[1] was prepared according to the method of Yagi et al. (1961) and purified by recrystallization from an ethanol–water mixture (Yagi et al., 1967). Its purity was checked by TLC (Sun et al., 1972). CCl_4 and acetonitrile were of spectro grade. For the photolysis experiment, CCl_4 was treated with methanolic KOH, washed with water, and distilled over P_2O_5. Cl_3AcOH, F_3AcOH, and BDEA were of reagent grade.

Spectral Measurements. Absorption and corrected fluorescence spectral measurements were made on a Cary 118C spectrophotometer, a Union Giken spectrophotometer, SM-401, and a Perkin-Elmer MPF3 spectrofluorometer.

Photolysis. The photoreduction of RFTB was carried out anaerobically by using BDEA as an electron donor. The photolysis mixture in CCl_4 that was placed in the cell compartment of a Hitachi Perkin-Elmer Model 139 spectrophotometer was irradiated with a high-intensity B & L monochromator and a 150-W Xe arc lamp, perpendicular to the monitoring beam, while the solution was slowly bubbled with oxygen-free N_2 prepared by passing prepurified N_2 through the 0.4 M $Cr(ClO_4)$-amalgamated Zn solution. The photoreduction was followed by an absorbance decrease at 440 nm with a simple flip of the light shutter. Prior to photolysis, the reaction mixture was flushed with oxygen-free N_2 for 40 min at 0 °C in the dark and was allowed to stand at room temperature until the temperature equilibrated. The actinometry of the photoirradiation system was carried out as described previously (Song & Moore, 1968; Sun & Song, 1973).

Results

The effect of hydrogen bonding on the absorption spectra of flavins was examined experimentally. First, the absorption spectrum of RFTB, which is soluble in organic solvents unlike naturally occurring flavins, was measured in CCl_4 (Figure 1).

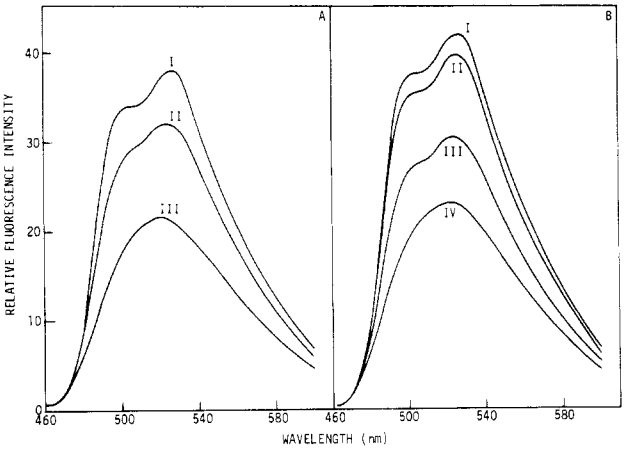


FIGURE 2: Effect of hydrogen bonding on the fluorescence spectrum of RFTB in CCl_4. Concentration of RFTB: 7×10^{-5} M. (A) Concentrations of Cl_3AcOH: 0 (I); 5×10^{-4} (II); 2×10^{-3} M (III). (B) Concentrations of F_3AcOH: 0 (I); 1×10^{-4} (II); 1×10^{-3} (III); 4×10^{-3} M (IV).

The flavin spectrum obtained in dry CCl_4 is essentially that of the non-hydrogen-bonded species. With increasing concentrations of Cl_3AcOH, the spectrum gradually changed as shown in Figure 1A; the first absorption band shifts slightly but definitely toward blue with an increase in absorbance, then shifts toward red, and finally locates at the original wavelength. On the other hand, the second absorption band shifts slightly toward red with a slight increase in absorbance and then shifts markedly toward red with a further increase in absorbance. The spectrum obtained with an excess amount of Cl_3AcOH is similar to that of riboflavin dissolved in water. When F_3AcOH was used instead of Cl_3AcOH, the spectral change shown in Figure 1B was observed. The change is essentially identical with that caused by Cl_3AcOH. This change cannot be ascribed to a change in the polarity of the medium, since the acids added were not sufficient to change the polarity of the bulk medium. It was further verified in this experiment that the addition of 0.1 M acetonitrile did not cause any significant change in the absorption spectrum.

Figure 2 shows the fluorescence spectra of RFTB in the presence of Cl_3AcOH or F_3AcOH. It can be seen that both Cl_3AcOH and F_3AcOH quench the fluorescence of RFTB, although the hydrogen-bonded species also fluoresce. It is noted that the fluorescence spectral shape changes with increasing concentrations of Cl_3AcOH or F_3AcOH, losing the characteristic vibrational shoulder at 503 nm. Fluorescence quenching constants for Cl_3AcOH– and F_3AcOH–RFTB

[1] Abbreviations used: RFTB, riboflavin 2′,3′,4′,5′-tetrabutyrate; TLC, thin-layer chromatography; CCl_4, carbon tetrachloride; Cl_3AcOH, trichloroacetic acid; F_3AcOH, trifluoroacetic acid; BDEA, *N*-benzyl-*N*,*N*′-dimethylethylenediamine.

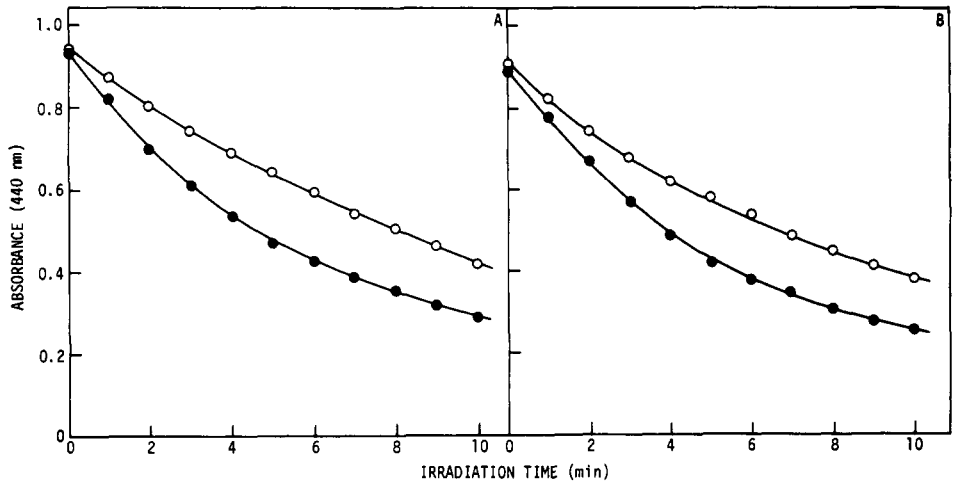


FIGURE 3: Effect of hydrogen bonding on photoreduction of RFTB with BDEA. Concentration of RFTB: 7×10^{-5} M. Concentration of BDEA: 1.4×10^{-3} M. Concentrations of Cl_3AcOH or F_3AcOH: 0 (○); 1×10^{-3} M (●). (A) Cl_3AcOH; (B) F_3AcOH.

Table I: Kinetic Data for the Photoreduction of RFTB in CCl_4 with BDEA in the Presence of Cl_3AcOH or F_3AcOH[a]

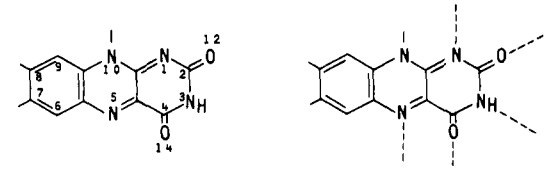
hydrogen-bonding donor (mM)	$\frac{-dA_{440}}{dt}_{t\to 0\,min} \pm$ 0.003/min[b]	photo-reduction[b] (%)	$\phi \times 10^2$ [c]	ϕ_{rel}
Cl_3AcOH: 0	0.067	~100	1.34	1.00
0.1	0.067	~100	1.34	1.00
0.5	0.080	~100	1.60	1.19
1.0	0.110	~100	2.20	1.64
2.0	0.096	~100	1.92	1.43
F_3AcOH: 0.1	0.071	~100	1.42	1.06
1.0	0.096	~100	1.92	1.43
4.0	0.078	~100	1.56	1.16

[a] Photoreduction was carried out with 1.4 mM BDEA, and each set of the data was run under identical conditions. [b] Evaluated from initial rates within 2–3 min of irradiation.[2] [c] Evaluated relative to the quantum yield (0.006) of photodecomposition of riboflavin in water (Sun & Song, 1973).

complexes were estimated to be 4.5×10^2 and 2.3×10^2 M^{-1}, respectively.

In order to ascertain the effect of hydrogen bonding on the reactivity of RFTB, we followed the photoreduction of RFTB with BDEA in the presence of Cl_3AcOH or F_3AcOH by monitoring the bleaching of RFTB as the result of the photoreduction of the flavin. Typical kinetic runs are shown in Figure 3. It can be seen that both Cl_3AcOH and F_3AcOH significantly enhance the initial rate of photoreduction. Table I summarizes the rate data. It should be mentioned that the quantum yields were estimated from the initial rates within 2–3 min of irradiation in order to minimize the error introduced by the decreasing intensity of absorbing light and quenching by products including semiquinones produced by disproportionation.[2] At high concentrations of Cl_3AcOH or F_3AcOH, the enhancement of the photoreduction rate decreases, as shown in Table I. In several runs, the maximum rate enhancement occurred with 1.4 mM BDEA and 1 mM Cl_3AcOH or F_3AcOH. The effects of Cl_3AcOH and F_3AcOH on photoreduction are not due to "pH" differences, as the solvent CCl_4 is a dried, aprotic solvent.

[2] A full recovery (100%) of the original absorbance (0.8–1.0) after air oxidation of the photobleached solutions ensured anaerobic conditions and suggests that the photoreduction was the predominant, if not exclusive, reaction, especially within the first 2–3 min of photoirradiation. A rough comparison indicated that the irreversible photodecomposition of RFTB was at least 2 orders of magnitude slower than photoreduction. After more than 30 min of irradiation, however, approximately 90% of the original absorbance was recovered after air oxidation of the photobleached solutions. It should be noted that RFTB is considerably more stable to light than riboflavin and flavin monophosphate because of the butylation of the ribityl hydroxyl groups which participate in the intramolecular photodecomposition reactions of the flavin moiety (Kurtin et al., 1967).



A. NO HYDROGEN BONDING B. N(1),O(12),N(3)H,O(14), N(5)-HYDROGEN BONDING

FIGURE 4: Hydrogen bonding at the heteroatoms of the isoalloxazine nucleus of flavin.

Discussion

The absorption spectrum of RFTB in CCl_4 (curve I, Figure 1) is considered to be that of the flavin with no hydrogen bonding (see Figure 4A). The spectrum of RFTB with excess Cl_3AcOH is similar to that of hydrogen-bonded species (Figure 4B). It is noted that the spectrum gradually changes with increasing concentrations of Cl_3AcOH (see curves I–III in Figure 1A). This indicates that hydrogen bonding of the flavin increases with increasing concentrations of the proton donor and, therefore, the affinities of the heteroatoms of the isoalloxazine to the proton donor are different from one another. The effect of F_3AcOH is essentially identical. This is also reflected in the fluorescence quenching constants (or apparent binding constants), the values of which are of the same order.

Since the proton affinities of the hetero atoms of the isoalloxazine are different from one another as mentioned above, the gradual change in the spectrum with increasing concentrations of Cl_3AcOH was compared with the previously reported data obtained by calculation (Nishimoto et al., 1978). When the presently observed shifts of the first and second absorption bands were compared with the calculated data shown in Figure 3 in our previous paper (Nishimoto et al., 1978), the sequence of hydrogen bonding can be assigned as follows: no hydrogen bonding → N(1)-hydrogen bonding → N(1),O(12)-hydrogen bonding → N(1),O(12),O(14)-hydrogen bonding → N(1),O(12),N(3)H,O(14)-hydrogen bonding → N(1),O(12),N(3)H,O(14),N(5)-hydrogen bonding.

The marked bathochromic shift of the second $\pi \rightarrow \pi^*$ band by hydrogen bonding is much higher than that of the first π

$\rightarrow \pi^*$ band (see curves I and III in Figure 1). This can be explained at least in part by stronger hydrogen bonding in the second π,π^* state (S_2) than in the lowest π,π^* state (S_1). In addition, the S_2 state is much more polar in its electronic structure. The difference between the S_0 and S_2 dipole moments (π moments) is calculated to be 10.49 D, which is greater than 5.4 D for the $S_0 - S_1$ difference in 7,8-dimethylisoalloxazine (P.-S. Song, unpublished data). The σ framework is not significantly altered by $\pi \rightarrow \pi^*$ excitation (Song, 1968). These features are consistent with the observation that hydrogen bonding exerts a stronger perturbation on the second absorption band than on the first absorption band.

In order to assess the effect of hydrogen bonding on the catalytic activity of the flavin nucleus, we adopted the photoreduction reactivity as a measure of the N(5) electrophilicity. This approach has been previously justified (Sun & Song, 1973; Song et al., 1976). In brief, it was shown that the photoreducibility of flavin was determined by the size of the LUMO (π^*) component of the $^3(\pi,\pi^*)$ state function at N(5). Thus, the photoreactivity of the triplet state is a measure of the electrophilicity of the ground state. Furthermore, the electronic structures of the ground and triplet states are similar for flavins, as reflected in the calculated dipole moments and the basicities of N(1) and N(5) (Song, 1971), while the structure of the excited singlet state is substantially different from those of the ground and triplet states. This trend appears to be general for many heterocyclic molecules (Jackson & Porter, 1961; Bertran et al., 1969; Song, 1980). In terms of perturbation theory, both photoreduction and nucleophilic addition at the N(5) of flavin involve the frontier orbital, π^*_r, as well as higher unoccupied orbitals. Protonation or hydrogen bonding at N(1) only slightly increases the size of π^*_r at N(5) (Sun & Song, 1973; Song & Sun, 1974; Nishimoto et al., 1978), but the N(5) reactivity increases substantially by protonation or hydrogen bonding at N(1) as the superdelocalizability at N(5) increases from 1.940 to 2.332 (Sun & Song, 1973; Song & Sun, 1974).[3] Hydrogen bonding at O(12) and O(14) increases the size of π^*_r even more (Nishimoto et al., 1978).

The concentration of Cl_3AcOH or F_3AcOH in our photoreduction experiment was adjusted to bring about hydrogen bonding at N(1), O(12), and O(14) but not at N(5). The data shown in Table I bear out the above theoretical prediction. It can be seen from Table I that the hydrogen bonding shows a definite rate enhancement, as was predicted by the theoretical calculations. At high concentrations of Cl_3AcOH or F_3AcOH, the rate tends to decrease. Since hydrogen bonding at N(5) decreases the size of π^*_r at N(5), the observed decrease in the rate could be ascribed to the occurrence of hydrogen bonding at N(5). Even though N(5) is the weakest heterocyclic nitrogen site of flavin in basicity in aqueous solution (Wyatt, 1976), hydrogen bonding seems to occur in dry CCl_4 at high concentrations of Cl_3AcOH or F_3AcOH. Otherwise, it might be explained by the formation of static quenching complexes between RFTB or BDEA and the acids. It should be noted that the rate enhancement observed up to 1 mM Cl_3AcOH or F_3AcOH is not due to an enhanced intersystem crossing to yield reactive triplet RFTB, since there is no direct correlation between fluorescence quenching (cf. Figure 2) and relative quantum yields (cf. Table I). Furthermore, at the concentration range used, Cl_3AcOH is a poor heavy atom perturber for intersystem crossing, and F_3AcOH cannot be an effective spin–orbit quencher. In analogy to our previous results (Sun & Song, 1973; Song et al., 1976), the photoreactive state for flavin photoreduction is a $^3(\pi,\pi^*)$ state.

From the foregoing considerations, the enhancement of photoreduction by Cl_3AcOH or F_3AcOH is most likely attributable to the effect of hydrogen bonding which increases the electron affinity of the flavin nucleus in general and the N(5) electrophilicity in particular.

In our previous study (Nishimoto et al., 1978), the calculated data coincided well with the spectral characteristics of crystalline flavodoxin from *Cl.* MP and from *D. vulgaris* (Mayhew & Ludwig, 1975). On the basis of these results, the spectral characteristics of D-amino acid oxidase were explained by the occurrence of hydrogen bonding at each nucleus of heteroatoms of the isoalloxazine except N(1). When we consider from the present results that the hydrogen bonding at N(1), O(12), N(3)H, and O(14) facilitates the reducibility of flavin, the hydrogen bonding involved in these flavoproteins is significant for their catalytic activity. This suggestion is of particular interest, since stacking interactions between the flavin nucleus and aromatic residues of the protein are counterproductive, as far as catalytic efficiency in nucleophilic attack and electron transfer at N(5) is concerned.

[3] The perturbation energy for nucleophilic attack at the rth position is given by the so-called superdelocalizability

$$SD^N_r = \sum_j^{unoccupied} (\pi^*_r)_j/-k_j$$

where π^*_r is the rth atomic orbital contribution to the unoccupied MO's (π^*) and k_j is the jth MO energy parameter (Fukui et al., 1957).

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Identification, Purification, and Characterization of Two Distinct Avian Vitellogenins†

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ABSTRACT: Chicken vitellogenin has been resolved into two species (VTG I and VTG II) by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Both vitellogenins are present in plasma from laying hens and estrogen-treated roosters, but neither is found in plasma from nonstimulated roosters. Amino acid and phosphorus analyses showed that VTG I and VTG II differed markedly in their content of serine, leucine, valine, and isoleucine but were indistinguishable in their phosphorus content. Comparison of VTG I and VTG II by limited proteolysis mapping yielded no evidence of similarity in either nonphosphorylated or phosphorylated peptides. Peptides generated through CNBr cleavage also showed no evidence of similarity. When tested by direct precipitin analysis or double-diffusion analysis, VTG I showed no evidence of reactivity with antibody raised against VTG II. We conclude from these data that VTG I and VTG II are physiologically relevant species which are products of distinct vitellogenin genes. The observation of VTG I and VTG II, without exception, in large numbers of birds from an inbred flock argues in favor of two vitellogenin genes within the haploid chromosome set. Comparison of lipovitellins and vitellogenins through limited proteolysis mapping yields evidence of a specific and complex relationship between these proteins. This analysis indicates that VTG II gives rise to polypeptides in both α-lipovitellin and β-lipovitellin while VTG I gives rise to only α-lipovitellin polypeptides. Several tentative assignments for specific polypeptides can be made. VTG II, for example, appears to be the precursor of the 125 000-dalton polypeptide common to both α-lipovitellin and β-lipovitellin. In addition, the intermediate molecular weight polypeptides which are unique to α-lipovitellin appear to derive from VTG I. Furthermore, calculation of vitellogenin phosphorus using amino acid analysis for the estimation of protein mass indicates that neither VTG I nor VTG II can give rise to both yolk phosvitins. The uncertainties in this type of calculation are discussed.

The hepatic synthesis of vitellogenin is regulated by estrogenic hormones in birds and amphibians as well as in other egg-laying vertebrates [for reviews, see Bergink et al. (1974) and Tata (1976)]. In the hen, vitellogenin is transported to the ovary and deposited in the yolk fluid after proteolytic cleavage to α-lipovitellin, β-lipovitellin, and phosvitins. Both lipovitellins contain two or more polypeptide chains which arise from regions of vitellogenin containing little or no phosphorus (Bergink et al., 1974; Bos, 1975; Jost et al., 1975; Deeley et al., 1975). The heavily phosphorylated regions of vitellogenin give rise to at least two phosvitins (Clark, 1970; Bergink et al., 1974; Christmann et al., 1977). It is of obvious importance for studies of vitellogenin regulation and vitellogenin structure to know whether vitellogenin is a unique protein. In the present study we report that chicken vitellogenin can be resolved into two species by $NaDodSO_4$[1]–polyacrylamide gel electrophoresis. The vitellogenins have been purified and compared on the basis of amino acid and phosphorus compositions, peptide maps, immunological reactivity, and their relationship to the yolk lipovitellins in order to evaluate the significance of this observation. The results of these comparisons lead us to conclude that the two vitellogenins are distinct gene products which serve as precursors to different lipovitellin polypeptides.

Experimental Procedures

Hormone Treatment, Isolation of Vitellogenin, and Lipovitellins. White leghorn roosters (SPAFAS, Norwich, CT) were injected intramuscularly with diethylstilbestrol (50 mg/kg) in propylene glycol on days 0 (primary), 4 (secondary), and 8 (tertiary) and bled 3 days after the indicated injection. Vitellogenin was isolated from plasma after secondary DES stimulation by the DEAE-cellulose procedure as described by Deeley et al. (1975) with three modifications. (1) Blood was drawn from the heart under pentobarbital anesthesia, and plasma was prepared as described (Williams, 1979). (2) The bulk of the plasma very low density lipoprotein was removed

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; VTG I, vitellogenin I; VTG II, vitellogenin II; diethylstilbestrol (DES), *trans*-α,α′-diethyl-4,4′-stilbenediol.

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