ELECTRON STAINS

I. Chemical Studies on the Interaction
of DNA with Uranyl Salts

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ABSTRACT

Chemical studies have been carried out on the interaction of DNA with uranyl salts. The effect of variations in pH, salt concentration, and structural integrity of the DNA on the stoichiometry of the salt-substrate complex have been investigated. At pH 3.5 DNA interacts with uranyl ions in low concentration yielding a substrate metal ion complex with a UO_2^{++}/P mole ratio of about $\frac{1}{2}$ and having a large association constant. At low pH's (about 2.3) the mole ratio decreases to about $\frac{1}{3}$. Destruction of the structural integrity of the DNA by heating in HCHO solutions leads to a similar drop in the amount of metal ion bound. Raising the pH above 3.5 leads to an apparent increase in binding as does increasing the concentration of the salt solution. This additional binding has a lower association constant. Under similar conditions DNA binds about seven times more uranyl ion than bovine serum albumin, indicating useful selectivity in staining for electron microscopy.

INTRODUCTION

During the last ten years the electron microscope has been used to study the structure of a vast number of biological materials. These studies have resulted in the accumulation of an enormous body of morphological information. However, very little cytochemical information has been obtained, although it has been frequently suggested that the chemical identification of tissue components could be accomplished by the use of suitable electron stains, that is, chemical substances which attach with some discrimination to the various chemical groups found in tissue sections and lead to a local increase in electron density.

The problem of electron staining and the necessary properties for a useful electron stain have been considered by a number of workers (9, 10, 23, 41). Isenberg (23) and more recently Valentine (41) have suggested, on the basis of theoretical arguments, that the organic dyes generally in use in histochemistry could be success-

fully used as electron stains. Some experimental support for their arguments has been obtained (15, 22). The recent analysis by Coslett of the requirements of electron staining makes clear that contrast in electron micrographs depends primarily on differences in density and thickness of different parts of the object under study (10). Hence, when considered on a weight basis, the efficiency of a stain depends on the weight of stain attached to the substrate. Clearly, if the stain contains heavy atoms, fewer molecules are required to cause a given increase in density. Early work by Hall (17) and Hall et al. (18) suggested the use of heavy metal ions as electron stains.

After testing the usefulness of a large number of heavy metal salts as electron stains, Gersh et al. (16) concluded that they were useful to improve contrast but not to give chemical information. Results obtained by other investigators, using as test substances various biological substrates in

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which some knowledge of the distribution of the component chemicals was available, have suggested that some specificity does occur. Bernstein (7) has shown that ferric ions could be successfully used to stain the nucleic acid in phage particles. Uranyl acetate has been used for the same purpose by Valentine (42), while Huxley and Zubay (21) used it to stain microsomal particles. Other heavy metal salts including uranyl nitrate, lanthanum nitrate, phosphotungstic acid, phosphomolybdic acid, cupric acetate, lead acetate, silver nitrate, and ferric nitrate have all been used as stains in the study of virus particles (25, 34, 37, 43, 44). In a somewhat different way silver salts have been used to stain DNA by a modified Feulgen procedure (24). Sikorski and Simpson (35) have used mercuric acetate to localize basic groups in keratin and have used both mercuric acetate and sodium plumbite to localize sulphydryl groups. Methyl-mercury chloride has been used for the same purpose by Bahr and Moberger (6). As a stain, osmic acid shows some specificity toward unsaturated lipids but as yet its reactions are not fully understood (1, 40). Protein has been selectively stained by a bromoacetic acid–lead method (26) and possibly also with mercuric bromphenol blue (19). Other staining methods employing heavy atom substituted organic compounds specific for protein have been reported (27, 36). In a number of studies glycogen has been stained by the PAS method or some modification of it (8, 30). In exploratory attempts to assess the general usefulness of heavy metal ions as electron stains Watson (44, 45) and Swift and Rasch (40) have examined tissue sections in the electron microscope after floating them on a variety of staining solutions. In a number of cases enhanced general contrast was obtained and in some instances useful specificity was indicated. However, as was pointed out by Watson (45), lack of knowledge concerning the chemical groupings for which the various metal ions had specific affinity demanded that caution be exercised in the interpretation of the results obtained. Thus it was not possible to draw precise inferences concerning the chemical nature of the structures emphasized by the staining.

In an attempt to obtain the chemical information crucial to a rational use of stains we decided to study the chemical interactions of selected metal salts with various substrates of biological origin. The suggestion that uranyl salts show some

selectivity toward nucleic acids encouraged us to direct our attention first to a chemical study of the interaction of DNA with uranyl acetate and uranyl nitrate solutions. The results of these studies together with some preliminary results on the staining of protein are presented in this paper.

A precise application of known staining reactions to practical cytochemistry requires a clear knowledge of the contrast effects of the stains on accurately definable materials. Concurrently with our chemical studies we have explored with the electron microscope the enhancement of contrast, as a result of staining, of isolated molecules of DNA. These results will be presented in a subsequent paper.

EXPERIMENTAL

Materials

DNA: The DNA solutions used in these investigations were prepared by dissolving commercially obtained DNA (highly polymerized, salmon sperm DNA supplied by the California Corporation, Los Angeles, for Biochemical Research) in 0.015 M NaCl at room temperature. After allowing the solution to stand several hours to facilitate complete dissolution the insoluble residue was removed by centrifugation. The solutions were stored at 0°C. The concentrations were then determined in two ways: (a) Aliquots were diluted as required with standard saline-citrate buffer solution (0.15 M NaCl, 0.015 Na-citrate) and the optical density determined at 260 mμ; or (b) one milliliter aliquots were evaporated to dryness, digested with 12 N HClO$_4$ for 1 hour in a boiling water bath (29), and then phosphate was determined directly by a modification of the method of Lowry *et al.* (28). Comparison of the results obtained by the two methods showed good agreement. Additional information concerning the characteristics of the sample was obtained by determination of the optical density of an aliquot before and after heating in a boiling water bath for 15 minutes. The two optical densities were 0.420 and 0.540, respectively. The increase in optical density of approximately 33 per cent is generally accepted as indicating native DNA. Analysis of a sample by sedimentation velocity in a Spinco analytical ultracentrifuge yielded an average molecular weight of approximately 2 million. This is in satisfactory agreement with results obtained by other workers for similar samples (14). Specimens prepared for observation

in the electron microscope showed primarily long thin strands with little evidence of denaturation.

Protein: The protein solutions were obtained by diluting serum albumin solutions (obtained from the Armour Pharmaceutical Company, Kankakee, Illinois) to the desired concentration with 0.15 M NaCl. Concentrations were then determined by means of the microburette method (46).

Uranyl Acetate: Uranyl acetate solutions were prepared gravimetrically from uranyl-acetate dihydrate (obtained from the J. T. Baker Chemical Company). Drying the dihydrate for 24 hours at 110°C was sufficient to remove the water of crystallization, but in general this was not done.

Uranyl Nitrate: Uranyl nitrate solutions were also prepared gravimetrically from uranyl nitrate hexahydrate (obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey, and from Fischer Scientific Company, Fair Lawn, New Jersey). The concentrations of these solutions were checked in two ways: (a) Measurement of the optical density at the absorption maximum of concentrated solutions and comparison of this value with the standard values given by Rodden and Warf (31); and (b) titration of dilute solutions with 0.1 N NaOH (31). Repeated tests showed that the deviations in the concentrations of the prepared solutions from the value determined by weighing were always less than 5 per cent, which is about the limit of accuracy of the analytical tests employed.

Methods

The quantity of uranyl ion bound by the DNA was determined by a simple analytical procedure. Solutions of DNA and the desired uranyl salt were mixed together after adjustment of their pH's to the appropriate value. A reference solution containing no DNA was prepared simultaneously. The DNA-uranyl complex precipitated at pH values less than about 5.5 and was removed by centrifugation. That the precipitation was complete was shown by measurement of the optical density at 260 mμ after removal of the uranyl salt by dialysis. After removal of the precipitate the quantity of uranyl ion remaining in solution was determined by complexing with ferrocyanide (31). The amount of complex formed was determined by measuring the optical density of the solution at 500 mμ on either a Beckman DU or Cary Model 11 recording spectrophotometer. Although the relation between the optical density at 500 mμ and the concentration of ferrocyanide complex was linear in the regions employed in this study, a reference sample with no DNA was run with every unknown. The difference in optical densities between the reference and unknown samples gave a direct measure of the quantity of uranyl ion bound by the substrate.

To determine the stoichiometry of the DNA-uranyl complex the following procedure was used (32). Solutions of a given concentration of substrate were added to solutions with varying amounts of uranyl salt. The number of moles of uranyl ion found free in solution after precipitation of the DNA-uranyl complex were plotted against the total number of moles of uranyl ion contained in the initial solution (see Fig. 4). The approximate stoichiometry of the DNA-uranyl complex is then given at the break in the curve. Also the strength of binding can be calculated from the shape of the curve using procedures given in reference 13.

For the protein solutions a modification of this method had to be used since the addition of the ferrocyanide caused the precipitation of the protein which had not been precipitated by the uranyl salt. In this case 10 ml of protein solution were placed in dialysis sacks (previously treated as recommended by Hughes and Klotz, 20) made from visking tubing (obtained from the Visking Division of the Union Carbide and Chemical Company, Chicago) and the sacks were placed in 15 × 120 mm lipless test tubes. After addition of the uranyl salt solution the tube was stoppered and shaken gently on a wrist-action shaker for 48 hours at room temperature. To eliminate Donnan effects all solutions were made 0.15 M in NaCl. The solutions were then analyzed by the ferrocyanide method as before.

A third method that was used for solutions of high concentrations of uranyl nitrate was a combination of the equilibrium-dialysis method with spectroscopic determination of the concentrations of the solutions. Investigation had shown (see following section) that the absorption was proportional to concentration for uranyl nitrate solutions at pH 3.6 in 0.15 M NaCl. Consequently, it was possible to determine the concentration of the solutions of unknown strength from the optical density and a standard curve. However, since the optical density varied with pH, standards were run with every sample. The optical densities

of the sample and the standard were then used as a measure of the uranyl salt remaining in the solutions. A comparison of the spectroscopic method with the ferrocyanide method on solutions of moderate concentration showed agreement to about 5 per cent.

Denaturation of DNA

Two solutions of denatured DNA were prepared for use in the staining studies. Fifteen ml of 36.3 per cent formaldehyde (J. T. Baker, analytical reagent) were added to 15 ml of DNA solution (5.5×10^{-3} M in P). The pH of the resulting solution (3.8) was adjusted to 3.5 and the solution was heated in a closed test tube for 30 minutes in a boiling water bath. One milliliter aliquots of the DNA solution were removed before and after heating and diluted to 25 ml with standard saline citrate solution after which the optical densities were determined. The values obtained were 0.88 and 1.27, respectively, an increase of 44 per cent in optical density.

RESULTS

Spectra of Uranyl Salt Solutions

Uranyl Nitrate: In Fig. 1 the spectra of a series of uranyl nitrate solutions are shown. The pH's given are the unadjusted pH's of the solutions as prepared. It is apparent from the figure that under the conditions of the experiment Beer's Law is obeyed. The fact that these solutions obey Beer's Law implies the presence of only a single ionic species. The highly ionized nature of nitrate salts in general and the inability of other investigators (4) to detect the presence of any complexing in uranyl nitrate solutions leave no doubt that the spectrum shown is that of the uranyl ion, UO_2^{++}.

At higher pH's the absorption maximum shifts to 430 mμ with a secondary maximum at 422 mμ, the three maxima at 427, 415, and 404 mμ no longer being observed (see Fig. 1). The pH range throughout which the gradual spectral shift occurs depends on the concentration of the uranyl nitrate solution and the presence of added electrolytes (*e.g.*, NaCl), the shift occurring at lower pH's in solutions of high concentration and high ionic strength. Spectra of solutions of uranyl nitrate in 0.15 M NaCl are similar to those just discussed. Titration of 4×10^{-3} M
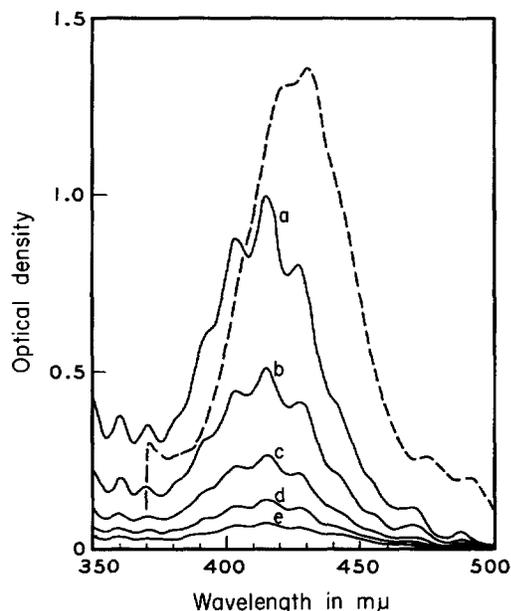

Figure 1

Spectra of uranyl nitrate solutions. The concentrations and pH's are as follows: *a*, 6 per cent, 1.75; *b*, 3 per cent, 1.8; *c*, 1.5 per cent, 1.9; *d*, 0.75 per cent, 2.1; *e*, 0.38 per cent, 2.2. The spectrum shown by the dotted line is for a 1 per cent solution at pH 5.0.

$UO_2(NO_3)_2$ and 4×10^{-3} M $UO_2(NO_3)_2$ in 0.15 M NaCl with 0.1 M NaOH revealed that precipitation occurs at pH's of 11.3 and 5.3, respectively. Previous investigation by other workers (2, 5) has shown that as the pH of uranyl nitrate solutions is raised hydrolysis occurs and polynuclear complexes are formed. At sufficiently high pH's these complexes aggregate and a visible precipitate results. The formation of these complexes is due to the reduction of the electrostatic charge by the addition of hydroxyl ions. A general formula of the form

$$UO_2[(OH)_2\,UO_2]n^{++} \text{ or } UO_2[OUO_2]n^{++},$$
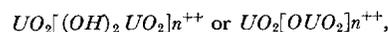
where *n* may assume any integral value, has been suggested for these complexes (5). Comparison of the spectral data and titration data obtained in the present investigation suggests that the spectral shift observed with increasing pH of the uranyl nitrate solutions is due to the formation of these polynuclear complexes.

Uranyl Acetate: The spectra of a series of uranyl

acetate solutions at different concentrations (Fig. 2) show that Beer's Law is not obeyed. Fig. 3 shows the variation of the absorption spectrum with pH. By comparison with Fig. 1 it is apparent that at low pH's the spectra of the acetate and nitrate solutions are identical. It follows that the absorbing species must be the same and that the acetate solution at low pH contains only uncomplexed uranyl ion. At higher pH's the uranyl ion is no doubt complexed but the nature of the complex has not been investigated in these studies. Other investigators (3, 11) have suggested that uranyl acetate complexes of the form UO_2Ac^+, UO_2Ac_2, and $UO_2Ac_3^-$ can be formed depending on the pH and concentration of
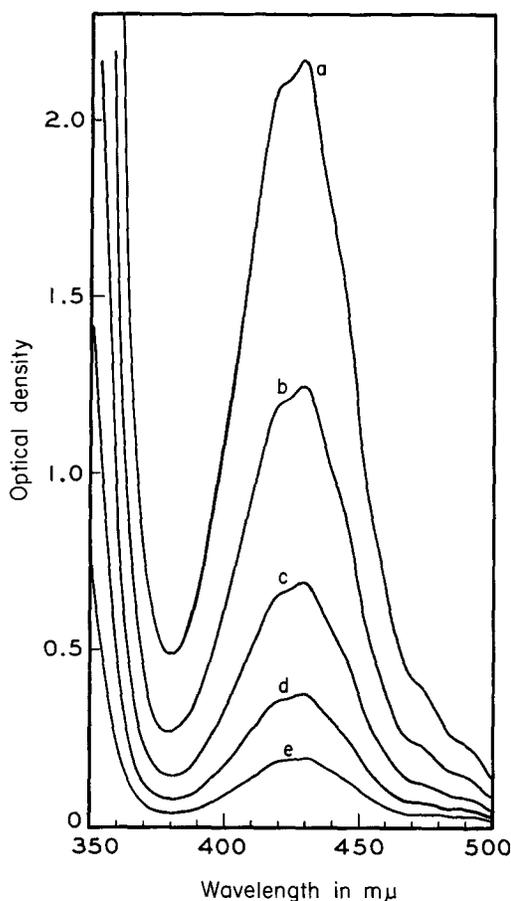

FIGURE 2

Spectra of uranyl acetate solutions. The concentrations and pH's are as follows: a, 3 per cent, 3.1; b, 1.5 per cent, 3.1; c, 0.75 per cent, 3.2; d, 0.38 per cent, 3.2; e, 0.19 per cent, 3.2.
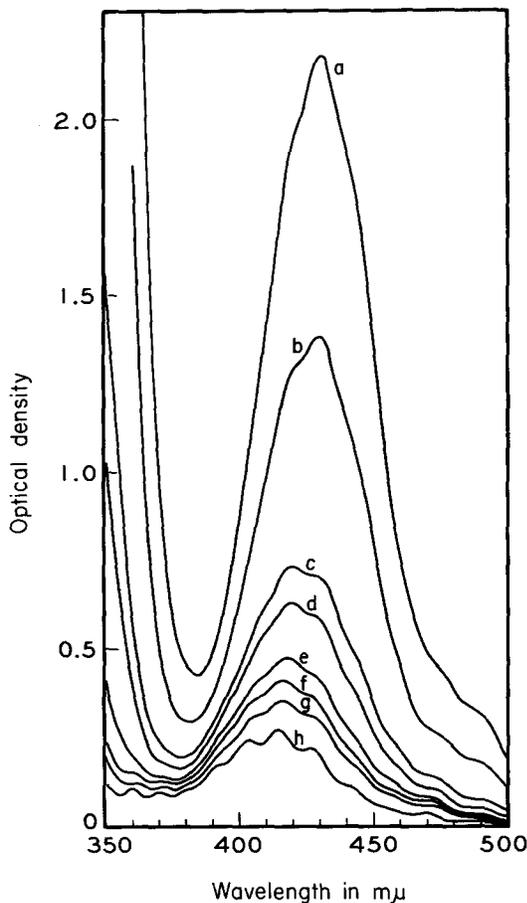

FIGURE 3

Variation of the absorption of uranyl acetate with pH. All spectra are for a 1.5 per cent solution, and the pH's are as follows: a, 5.1, b, 4.5, c, 3.9, d, 3.6, e, 3.4, f, 3.3, g, 3.0, h, 2.1.

the acetate ion present. That hydrolysis does occur in solutions of pure uranyl acetate (i.e., no acetate buffer) and is the predominant effect at higher pH's is suggested by the similarity of the spectra of uranyl acetate and uranyl nitrate under these conditions (see Figs. 1 and 3). As in the case of the uranyl nitrate solutions, the pH range, over which this transition from uncomplexed to complexed uranyl ion occurs, varies with concentration and the presence of added electroytes. At higher concentrations of uranyl acetate or in the presence of added electrolyte the transition occurs at lower pH's than in solutions of lower ionic strength.

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Qualitative Behavior of DNA in Staining Solutions

When DNA was added to either uranyl acetate or uranyl nitrate at low pH, a white precipitate was formed. The presence of NaCl had no observable effect on the ability of these uranyl salts to cause the precipitation of DNA. The precipitate was apparently stable over the pH range of 0.2 to about 6, but above pH 6 it went into solution. The addition of acetate or citrate buffers to a solution containing the precipitated DNA–uranyl complex also caused it to go into solution. Since both acetate and citrate form complexes with uranyl ion they presumably cause dissolution of the DNA–uranyl complex by removal of uranyl ions.

Staining of DNA with Uranyl Salts

In Table I the results of a number of staining experiments are given. The detailed data from one experiment are shown in Fig. 4. In all of these experiments the stoichiometric ratio of uranyl ions to phosphorus in the complex was deduced by the extrapolation method described above. The estimated error in these values is less than 10 per cent.

It is clear from the data that when uranyl nitrate is used at pH 3.5 one uranyl ion is bound for every two phosphate groups. This ratio is consistent with the view that uranyl forms a simple salt with the phosphate groups on DNA. When uranyl acetate is used at pH 3.5 the stoichiometric ratio is apparently slightly reduced.

If the pH is lowered to about 2.3, or if the DNA is treated by heating in the presence of formaldehyde, the stoichiometric ratio is reduced to one uranyl ion for every three phosphate groups.

The very sharp break typified in Fig. 4 indicates a strong association between uranyl ion and DNA. By utilizing the data from experiments XIX, XXXI, and XXXIII it was possible to determine an approximate association constant (Ka $\simeq$ 8 $\times 10^6$) for the DNA-uranyl complex. This value may be compared with an association constant of 2 $\times$ 10^5 found for a DNA-magnesium complex by Shack and Bynum (33). The uranyl ion is apparently more tightly bound than the magnesium ion. It should be noted, however, that the approximate association constant determined above is applicable only to the specific binding at pH 3.5 and in the narrow range of concentrations studied. At higher concentrations of uranyl ion or at higher pH's an additional looser binding seems to occur and an appropriately modified association constant would be required to describe the resulting complex. The experiments of Shack and Bynum were carried out at considerably higher pH's (9.4 and 10.2) and a greater range of ion concentrations was employed.

If the association constant is large, then in an excess of uranyl ion the number of unoccupied sites on the DNA is small and the stoichiometric ratio is very nearly equal to the ratio of uranyl ions bound to the number of phosphate groups in the DNA. If these conditions do not prevail, then the ratio of uranyl ions bound to phosphate

TABLE I

Staining of DNA with Uranyl Salt Solutions Over the Range 1 $\times$ 10^{-4} to 1 $\times$ 10^{-3} M

No. of Experiment	Stain	Moles $\times$ 10^6 DNA-P	pH	Moles $\times$ 10^6 UO_2^{++} bound	Mole ratio UO_2^{++}/P
XIX	$UO_2(NO_3)_2$	6.1	3.6	3.00	0.49
XXXI	"	5.5	3.5	2.60	0.47
XXXIII‡	"	5.5	3.5	2.53	0.46
XXVIII	"	6.3	2.2	2.05	0.33
XXXV	"	5.5	2.4	1.93	0.36
XXXVI	"	5.5	2.3	1.87	0.34
XXXVII*	"	5.5	3.5	1.75	0.32
XXXIX*	"	5.5	3.5	1.94	0.35
XXIV	$UO_2(C_2H_3O_2)_2$	6.3	3.5	2.80	0.45
XXIX	"	6.3	3.5	2.60	0.42
XXX	"	6.3	3.5	2.50	0.40
XXVII	"	6.3	2.4	1.85	0.29

* The DNA used in these experiments was denatured as described in the text.
‡ The data from experiment No. XXXIII are shown in detail in Fig. 4.

groups, obtained experimentally, will be less than the true stoichiometric value. The amount of uranyl attached to the DNA can be obtained by subtracting, from the total quantity of uranyl added to the solution used in the experiment, the amount of uranyl left free after the addition of DNA. In this way an approximate stoichiometric ratio can be determined from measurements at a single concentration. The results of several such experiments are given in Table II. The numbers listed in columns six and seven are average values obtained from four identical experi-
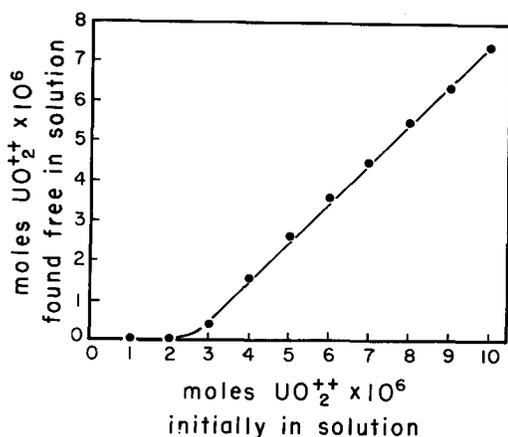

FIGURE 4

Staining of DNA with uranyl nitrate at pH 3.5. The intercept of the straight line portion of the curve on the x-axis gives the approximate stoichiometric ratio of the DNA-uranyl complex. Data from Experiment XXXIII (see Table I).

ments. In these experiments, carried out at higher pH's, the addition of ferrocyanide to the solutions occasionally caused the solution to become slightly turbid. For this reason the results obtained in these particular experiments are more variable and less reliable. Within the limitations just pointed out, however, the data in Table II indicate an enhanced attachment of uranyl ion to DNA at higher pH's.

In attempts to explore further the staining at higher pH's, two experiments were run with concentrations of uranyl ion varying from 0.7×10^{-3} to 4×10^{-3} M. A plot of the moles of UO_2^{++} found free in solution vs. the moles of UO_2^{++} added is shown in Fig. 5. There is not a clear sharp break in the curve as previously.

In addition to the experiments listed above, a number of experiments were carried out using high concentrations of uranyl salts and the equilibrium-dialysis technique. In these experiments the relevant concentrations were determined spectroscopically. Since the numbers of interest are the differences between two large numbers, there is considerable variation in the results obtained. The data in this case (see Table III) suggest a tendency toward greater uptake of salt from the solutions at the higher concentrations of uranyl nitrate. The slow increase in binding with increase of uranyl ion concentration indicates that the association constant for this additional weak attachment is very much lower than for the strong binding mentioned earlier. No attempts have been made to discover the stoichiometry of this weaker binding.

TABLE II

Staining of DNA with Uranyl Salt Solutions at Fixed Concentrations

The concentration of the staining solutions used in these experiments was approximately 2×10^{-4} molar.

No. of Experiment	Stain	Moles $\times$ 10^6 UO_2^{++} added	Moles $\times$ 10^6 DNA-P	pH	Moles $\times$ 10^6 UO_2^{++} bound	Mole ratio UO_2^{++}/P
VIA	$UO_2(NO_3)_2$	20	4.24	3.7	2.3	0.53
VIB	"	10	4.24	3.7	2.04	0.48
VII	"	20	4.24	5.2	11.15	2.63
IX	"	20	4.24	5.0	11.0	2.60
X	"	20	4.24	5.0	7.3	1.72
XI	"	20	4.24	4.8	5.0	1.18
XII	"	20	4.24	4.7	6.2	1.46
XX	"	16	6.1	5.8	14.7	2.42
XXI	"	18	6.1	5.8	13.5	2.22
XV	$UO_2(C_2H_3O_2)_2$	20	4.24	5.5	11.0	2.60

TABLE III

Staining of DNA with Concentrated Solutions of Uranyl Nitrate

Concentration of UO_2^{++} moles/liter	Mole ratio UO_2^{++}/P
6.0×10^{-2}	1.54 ± 0.19
$4.8 \times$ "	1.3 ± 0.20
$3.6 \times$ "	1.41 ± 0.20
$3.0 \times$ "	1.26 ± 0.16
$2.4 \times$ "	1.2 ± 0.12
$1.2 \times$ "	0.92 ± 0.02
$1.0 \times$ "	0.73 ± 0.07

Results of Preliminary Studies with Proteins

Qualitative behavior of bovine serum albumin in staining solutions. Qualitative studies of the nature of the interaction of protein with uranyl salts, analogous to those carried out with DNA, were done. The protein was also precipitated by uranyl salts. The presence or absence of sodium chloride had no observable effect on the precipitation of the protein by uranyl salts and, as before, uranyl acetate and uranyl nitrate behaved similarly. (Note, however, that Dounce et al. (12) report that BSA is not precipitated by uranyl acetate in the absence of salt.) In contrast to the results obtained with DNA, the protein-uranyl precipitate was soluble in strongly acidic solutions as well as strongly basic ones. The precipitate was stable only in the pH range of about 4 to 6. Here, again, it was found that the precipitate could be formed or dissolved repeatedly by adjustment of the pH. This was true in both uranyl acetate and uranyl nitrate solutions. It should be pointed out

that these results are essentially in agreement with those obtained by Dounce et al. in a series of similar investigations. As with the DNA-salt complex, the protein-uranyl complex was soluble in buffer solutions of citrate and acetate.

Staining of Protein: A combination of the equilibrium dialysis technique with ferrocyanide analysis was used to determine the stoichiometry of the uranyl-protein reaction. However, instead of varying the uranyl concentration, the protein concentration was varied. As can be seen from the data plotted in Fig. 6, a linear relation was found between the moles of bound uranyl ion and the amount of protein added. The slope of the line corresponds to 2.0×10^{-7} moles UO_2^{++} bound/mg of protein. In a similar experiment, only using varying concentrations of uranyl nitrate, Rothstein and Meier (32) found that 2.5×10^{-7} moles of uranyl ion were bound/mg of protein.

DISCUSSION

Two attributes desirable in electron stains are: (a) The ability to scatter electrons appreciably, and (b) the ability to attach extensively and reproducibly to specific chemical groups. Uranyl ions, or complex ions involving the uranyl group, contain an atom of high atomic number which is very effective in scattering electrons. Thus they readily fulfill one of the requirements of an electron stain.

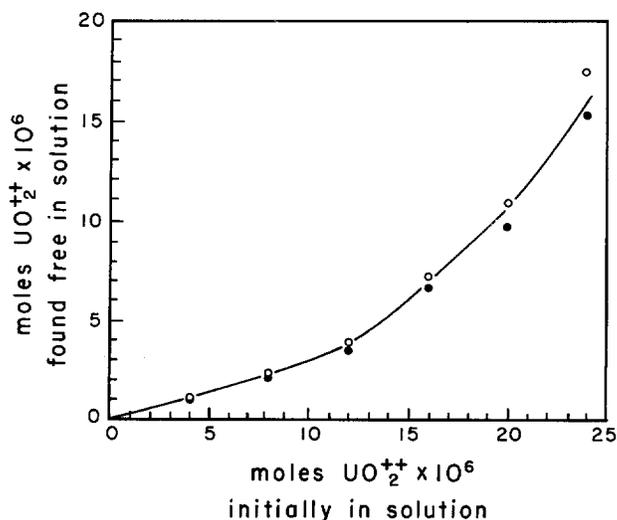The stoichiometric relations found in these

FIGURE 5

Staining of DNA with uranyl nitrate at about pH 5. The absence of a curve with a sharp break indicates weak or multiple site binding (see text). The data from two independent experiments are represented by the full and empty circles.

investigations indicate that at pH 3.5 one uranyl ion is attached for every two phosphate groups in DNA. In a subsequent paper it will be shown that the staining leads to a very noticeable increase in the contrast. This contrast is adequate for the visualization of strands of DNA.

The attachment of uranyl to DNA is very strong as is clearly shown by the large association constant or the sharp break in the curve of Fig. 4. At higher pH's additional binding does take place, but the association constant for the complex is weaker. This is shown by the much less abrupt break in the curve of Fig. 5. Strong attachment with a precise stoichiometry is a most desirable feature of stains in that it ensures reproducibility.

The crucial question of selectivity can be answered in part by a comparison of the results obtained in the staining of DNA with those obtained in the staining of BSA. Comparison of the data presented in Table I and Fig. 6 reveals that at pH 3.5 and on an equal weight basis the DNA is stained about seven times as heavily as the protein. This is known to be true only for BSA, the particular protein used in these studies. Since, no doubt, the binding of uranyl to protein varies with the charge on the protein, other proteins can be expected to bind more or less uranyl. In particular, phosphoproteins may stain readily with uranyl salts. A more thorough study of the binding of uranyl ions by proteins is now in progress. Nevertheless, the data presented here

do give an indication of the extent to which differential staining may be obtained under favorable conditions. The sevenfold preference of uranyl for DNA over protein promises a discrimination of considerable use in electron microscopy. Selectivity in staining by uranyl has also been pointed out by Valentine (42) and Huxley and Zubay (21) although no precise quantitative data have been previously given.

The attachment of stain to DNA at pH 2.2 and 3.5 is only slightly greater from uranyl nitrate solutions than from uranyl acetate solutions. This is readily seen in Table I. That the efficiency of staining of the two salts is very nearly the same under the conditions employed in these studies is not surprising in view of our spectroscopic investigations which suggested that the ionic species in the solutions are probably also essentially the same. When high concentrations of stain are used to stain tissue sections (e.g., 2 per cent or greater, see Swift (39), Watson (44), etc.) and non-specific staining occurs, it may very well be that the acetate salt and nitrate salt will not be of equal efficiency as electron stains. The primary difference between solutions of uranyl acetate and uranyl nitrate at higher concentrations (as well as at higher pH's) is that the acetate solution may contain the monovalent ion, UO_2Ac^+, in addition to polynuclear complexes of uranyl and hydroxyl ions, whereas the nitrate solution will contain uranyl ions or possibly polynuclear com-
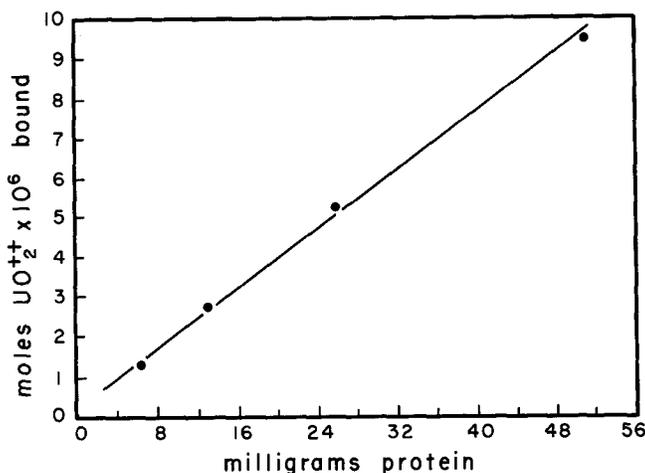

FIGURE 6

Binding of uranyl ion by bovine serum albumin. The stoichiometry of the binding is given by the slope of the curve (see text).

plexes of uranyl and hydroxyl ions. It is conceivable that in moderately rigid systems such as fixed and embedded tissue sections, the monovalent stain would be more efficient than a divalent one.

On increasing the pH, the stain removed from solution by the DNA appears to increase rapidly also, as shown in Table II. However, in this region it is difficult to obtain reproducible values of the extent of staining. Furthermore, at pH's only slightly higher than pH 5 a precipitate forms in the staining solution even without DNA. Under these conditions very weak associations with the substrate would lead to additional precipitation of the stain. Raising the pH increases the effective charge on the DNA molecule making possible the binding of additional metal ions: it also gives rise to the formation of polynuclear complexes of uranyl and hydroxyl ions which, if bound, would result in a similar increase in the amount of stain attached to the DNA. Such binding would be weaker than the highly specific binding at pH 3.5 and, consequently, a sharp break in the line relating the metal ion found to metal ion added would not be expected. The data plotted in Fig. 5 are consistent with this interpretation. Such weak association would be encountered in different chemical groups and the selectivity of the stain would be decreased. The data in Table II also suggest that there is a real increase in binding with increase in pH above 3.5. A molar UO_2^{++}/P ratio of the order of 1/1 or more is indicated by the data in the table at higher pH's. This increase in binding with increasing pH is in agreement with the behavior displayed in other systems of uranyl ions and organic substrates. Rothstein and Meier (32) have previously shown that complexes of uranyl ions with polyphosphates have increasing stability at higher pH's. Similar results have been reported for complexes of uranyl with organic acids (11). The abrupt decrease in apparent staining that occurs when the pH is decreased from 3.5 to about 2.2 appears to be primarily a consequence of the structural changes which the molecules undergo on acidification of the solution (although changes in the degree of ionization of the phosphate and base groups may also be involved). The importance of the structural alterations is shown by the fact that the same decrease in staining can be obtained at pH 3.5 if the DNA is disrupted by heating in the presence of formaldehyde prior to staining. That a con-siderable change in structure occurs during this treatment is indicated by the large increase in optical density that is observed. Whether the attendant decrease in staining is due to the intramolecular steric changes involved, the entanglement of the molecules, or occurs because some of the phosphate groups are neutralized by the free base amine groups, cannot be determined from the data obtained.

At higher concentrations of uranyl salt an increased attachment of cation occurs. Although the site of binding of the uranyl to the DNA is not known, it does not seem likely that the increased attachment at these higher concentrations is as specific as that at the lower concentrations in which a precise stoichiometry and a very high association constant can be determined.

The actual nature of the ligand-substrate complex has not been determined in these studies. However, studies of the reaction of uranyl acetate with protein solutions have suggested that the binding is due primarily to carboxyl groups (11, 32). Since both egg albumin and serum albumin bind enough uranyl ions to account for only about ⅕ of their free carboxyl groups (32) it seems likely that adjacent accessory groups are involved in the binding. The enhanced reactivity of associated groups is also suggested by the more effective complexing of uranyl by citrate than by acetate buffers. In an investigation of the complexing ability of a series of phosphates Rothstein and Meier (32) found that the mole ratio of uranyl bound to phosphorus increased regularly with increasing size of linear phosphate polymers. This mole ratio was essentially the same for highly polymerized polyphosphates and DNA. This suggests that the uranyl is bound at the phosphate locus in such compounds. From an examination of the infrared spectra of stained and unstained DNA we have found that only in the region of 900 to 1300 cm^{-1} were any shifts observed in the positions of the bands. Since all the bands in this region have been assigned to vibrations of the phosphate or sugar groups (38), it seems likely that the uranyl is attached at this site. No perturbations were observed in bands at higher frequencies which are associated with vibrations of the bases and more particularly of the amine and carbonyl groups.

Finally, we would draw attention to the necessity of a careful investigation into the reaction of biological substrates with heavy metal ions that

are of potential use as electron stains. Under the conditions studied here a visible precipitate is formed when DNA reacts with uranyl at low pH's. But at higher pH's or in the case of protein this is not true. The ferrocyanide test showed that uranyl ions are taken up by DNA at higher pH's and also by protein, but no observable change was apparent. Such a result makes clear the limitations of rough qualitative tests as indications for the suitability of various metal ions as electron stains. The variation in the extent of binding with pH also suggests that the evaluation of the potential usefulness of metal ions as electron stains by their behavior at a single pH may lead to conclusions of limited validity.

The information obtained in these studies is insufficient in itself to prove the usefulness of uranyl salts as electron stains. Consequently, simultaneously with the chemical studies we have been carrying out cytological studies to determine the effect of staining DNA with uranyl salts (the results of these investigations are to be described in more detail in a subsequent paper). Strands of DNA have been stained and compared with unstained samples by observation in the electron microscope. The increase in contrast due to staining is clearly apparent.

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