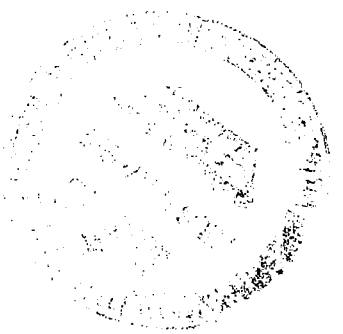
NASA CONTRACTOR REPORT

EXPERIMENTAL STUDIES FOR THE DETECTION OF PROTEIN IN TRACE AMOUNTS

by E. R. Walwick and B. R. Zalite

Prepared under Contract No. NASw-770 *by*
AERONUTRONIC
Newport Beach, Calif.
for



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ABSTRACT

Further research is presented on a program to demonstrate the utility of the dye, 4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine,for the detection of biological macromolecules. Analysis of stoichiometry studies on the reaction of the dye with various macromolecules were carried out and they indicate that for optimal reaction a one to one ratio of dye to anion site on the polymer is generally required to form the various complex states. These states correspond roughly with spectral band maxima at 570, 535, 510, 650 and 470 mμ. Titration of materials giving a J-band (650 mμ peak) indicated that this state arises through reaction of individual dye molecules with particular sites as a function of dye configuration and conformation to the site rather than being due to dye-dye interaction in a very large aggregate of dye molecules as previously supposed.

Results from reaction of the dye with soil extracts are presented. Inorganic ions and aluminosilicate minerals which might interfere with the dye reaction were investigated and procedures were developed to circumvent interference. This involved the testing and selection of a soil processing procedure which permitted removal of the inorganic materials from, and retention of the humic acid by, the soil extract. Reaction of the dye with humic acid from soil was investigated by titration of the humic acid with the dye, and determination of functional groups by titration and chemical modification.

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SECTION 1

INTRODUCTION

This report is the third in a series on a program designed to demonstrate the utility of the dye, 4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine, for the detection of biological macromolecules. This report presents results on the mechanism of the dye reaction, methods of eliminating inorganic materials which react with the dye, detection of humic acid with the dye and the nature of this reaction.

Initial work was concerned with reactivity of the dye with a wide variety of materials. This consisted of testing inorganic salts, polypeptides, simple proteins, conjugated proteins, microorganisms, synthetic polypeptides, polynucleotides, carbohydrates, amino acids, pyrimidine and purine bases, nucleosides, nucleotides and inorganics for their reactivity with the dye. In trace amounts (less than 0.002 percent) only proteins, synthetic polypeptides, nucleic acids, microorganisms, pollen and substituted polysaccharides caused changes in the absorption spectrum of the dye. Mono, di, and trisaccharides, purine and pyrimidine bases, amino acids and nucleosides had no effect. Polypeptides and nucleotides were effective only at higher concentrations. The action of inorganic salts depended on the nature of the anion. The influence of such variables as pH, temperature and dye-macromolecule stoichiometry on the stability and formation of the dye-macromolecule complexes was also determined. The optimum conditions for dye-macromolecule formation and stability appeared to be at a pH of 7-9, a dye concentration of 10^{-5} M and a temperature of 20-40^{o}C. This work reported in detail, both in the final report (Kay, 1963) and in two technical papers (Kay, et al., 1964a; Kay, et al., 1964b), demonstrated the utility of this dye for the detection of biological macromolecules. The initial study was proposed to determine the applicability of utilizing the dye to detect proteins. However, the spectral changes obtained upon

reaction of the dye with other biological molecules made it apparent that other important classes of biological macromolecules such as nucleic acids, and substituted polysaccharides could also be included. The reaction of the dye with these materials varied with the macromolecule tested and absorption peaks could be obtained at 575, 535, 510, 480 and/or 650 mμ. Because some proteins reacted with the biological macromolecules to give the formation of a sharp absorption peak at 650 mμ, which is known as a "J-band", the program became known as the "J-band Life Detector".

Subsequent work with the dye has dealt with the stoichiometry of the reaction and the mechanism of the dye reaction. This work has been prepared and accepted for publication. This paper is presented largely intact as Section 3.1 of this report and constitutes a considerable contribution to the understanding of the mechanism of such dye reactions. This work has shown that for optimal reaction a one to one ratio of dye to anion site on the polymer is generally required in the formation of the various complex states which correspond roughly with spectral band maxima at 570, 535, 510, 650, and 470 mμ. Little or no shift of spectral maxima for the complexes was found to occur with large ratios of sites to dye molecules. Titration of materials giving a J-band (650 mμ peak) indicates that this state arises through reaction of individual dye molecules with particular sites as a function of dye configuration and conformation to the site rather than being due to dye-dye interaction in a very large aggregate of dye molecules as previously supposed.

Much of this work on the mechanism was carried out with comparatively well defined biopolymers. These included pectins with varying degrees of methylation, alginic acid, poly-L-glutamate, poly-L-aspartic acid, bovine pancreas ribonuclease-oxidized, poly-L-lysine, horse heart hemoglobin, horseheart myoglobin, deoxyribonucleic acid, α-chymotrypsin, α-chymotrypsinogen, pepsinogen, pepsin, hyaluronic acid, polygalacturonic acid and metaphosphate.

Since any sample extracted from soil is likely to be composed of a mixture of various types of macromolecules, it was important to determine the behavior of the dye in known mixtures to obtain information on the relative effectiveness of individual polymers and upon possible effects of macromolecule interactions. Tests were made upon mixtures of polysaccharides, proteins, nucleic acids and inorganic polymers.

Inorganic compounds were chosen for test because their chemical nature indicated the possibility of reaction with the dye. In a biological detection scheme it would be necessary to exclude such reactions, since they would constitute false positive information. The inorganic materials chosen for this study constituted a broad spectrum of inorganic chemical types, which could interfere with the dye-organic compound reaction. However, this work demonstrated that these materials can be removed by processes normally employed for soil extraction.

Humic acid has been extracted from soils by a variety of extractants. These have included sodium pyrophosphate, sodium ethylenediaminetetra acetic acid, lactic acid, sodium sulfite, water and sodium hydroxide. In addition to employing these materials as extractants they have, in some cases, been employed for pretreatment prior to sodium hydroxide extraction. In all cases, these pretreatments were employed to remove divalent ions, and thus improve extraction of organic material. In recent work, extraction has been preceded with a mixture of hydrofluoric and hydrochloric acids, to obtain solution of some of the silicate material.

An important problem in this work has been to demonstrate that the dye was reacting with humic acid and not some trace of inorganic material. One approach included fractionation of humic acid on DEAE modified cellulose columns. The fractions obtained from the column were analyzed for carbohydrate, protein, uronic acids and reaction with the dye. Some fractionation was obtained. In several cases, a soil component was isolated which gave an absorption peak of 650 mμ with the dye instead of the 535 mμ peak seen with unfractionated soil samples and the other soil components from the fractionation. Amino acids and monosaccharides were demonstrated to be constituents of most of the fractions containing soil extract materials. Size distribution of the humic acids was studied by means of Sephadex gel filtration columns. However, little fractionation was seen. In most instances the soil components were either excluded by the column or appeared as a single component of small molecular weight material. Much of this work appeared in the second report of the series (Bean, et al. 1964). In initial studies the presence of inorganic materials in humic acid preparations was determined by incineration of the soil sample or soil extracts. In all cases the results indicated an absence of interference from inorganic materials. However, since modification of silicates could occur under these heating conditions, leading to the false assumption that no soluble silicates were present, other procedures were pursued. These included demonstration of the removal of various purified silicates and alumino-silicates by alkaline dialysis and following the decrease of silicate in soil extracts by means of a silicomolybdate complex. Acetylation and methylation of the humic acid have been investigated as a means of demonstrating that the dye is reacting with the humic acid functional groups. Carboxyl group concentration of the humic acid was determined by the calcium acetate method (Blom, et al., 1957), to permit correlation of carboxyl groups with dye reactivity.

SECTION 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 THIACARBOCYANINE DYE

Two samples of the dye, DBTC, were gifts of F. W. Mueller, Ansco, and J. A. Leermakers, Eastman Kodak Company. An additional sample was obtained commercially from Gallard-Schlesinger Chemical Manufacturing Corporation. All operations during the preparation of the stock solutions (1.2×10^{-4}M) were carried out in the dark or in minimal light to avoid photodecomposition of the dye. Stock and diluted working solutions were stored in brown polyethylene bottles, wrapped in aluminum foil, since the dye is readily adsorbed upon glass.

2.1.2 BIOPOLYMERS

We are indebted to K. Wilson and C. Smit, of Sunkist Corporation, for the special pectin and polygalacturonic acid samples used in these studies. Their uniform methoxyl pectin fractions (7.0, 9.4, 10.8 and 11.2 percent methoxyl) were isolated by ion exchange fractionation of natural citrus pectin. Samples with enriched methoxyl (above 11.3 percent) were obtained by chemical esterification of citrus pectins, either by methylation with diazomethane, or by acid catalyzed esterification in methanol.

Methyl alginate was prepared by acid catalyzed esterification of alginic acid. The alginic acid and sodium alginate were purified from a commercial sodium alginate (Matheson, Coleman and Bell) by repeated solvent precipitation.

Other preparations were obtained from commercial sources as follows: poly-L-glutamic acid, sodium salt, and poly-L-aspartic acid from Sigma Chemical Company; bovine pancreas ribonuclease (5x crystallized, salt free), oxidized ribonuclease and poly-L-lysine hydrobromide from Mann Research Laboratories; bovine albumin (crystalline), protamine sulfate (clupeine), glycoprotein, human hemoglobin (2x crystallized) and horseheart myoglobin from Nutritional Biochemicals Corporation; deoxyribonucleic acid, highly polymerized, α-chymotrypsin (3x crystallized), pepsinogen, α-chymotrypsinogen, pepsin (2x crystallized) from Worthington Biochemicals Corporation; chondroitin sulfate (sodium salt), hyaluronic acid (potassium salt), and polygalacturonic acid, from California Corporation for Biochemical Research; sodium polymetaphosphate, average degree of polymerization 18.5, from Victor Chemical Works.

2.1.3 MINERALS

The following minerals were obtained from Ward's Natural Science Establishment, Inc.: a) Limonite, Tuscaloosa County, Alabama, b) Limonite, var. yellow ocher, Massive (2 Fe_2O_3.3 H_2O) near Cartersville, Georgia, c) Montmorillonite (Bentonite), Clay Spur, Wyoming, d) Amphibole (Uralite), Calumet, Colorado, and e) Phlogopite ($K_4Mg_{12}Al_4Si_{12}O_{40}$ $(OH)_8$) Quebec, Canada. Silicic acid (SiO_2.nH_2O assay 77.8% as SiO_2) was obtained from the J. T. Baker Chemical Company. Two soluble silicates of composition: K_2O/SiO_2, 1:2.10 (w/w) and Na_2O/SiO_2, 1:2.86 were obtained from Victor Chemical Works.

2.1.4 SOILS

Two soils were utilized for the majority of the work reported here. One soil was a Mohave Desert soil obtained from Dr. R. E. Cameron of JPL, Pasadena, California, and designated soil 76-2. The other was a local agricultural soil located near the coast and containing a considerable quantity of clay materials and designated Mesa Verde. Additional soils were reported previously (Bean et al., 1964) and were obtained from the University of California, Riverside, or from Dr. Cameron at JPL.

2.1.5 BUFFERS

The spectra of the dye and its complexes are affected by high ionic strength so buffers were used at 0.001 M to 0.002 M final concentration, unless otherwise specified. At such concentrations, none of the normal organic buffering anions had significant effect and so it was possible to choose the most convenient buffer for obtaining the desired pH.

2.2 METHODS

2.2.1 TITRATION PROCEDURE

The following procedure was utilized for all titrations reported here. An appropriate portion of the biopolymer solution, the buffer and sufficient water to make a standard volume were mixed in a spectrophotometer cuvette. The dye was then added, the solution mixed and the absorption spectrum determined by scanning from 700 to 400 mμ in a Beckman DK-2A recording spectrophotometer. For each titration point, a new mixture was prepared at a different polymer concentration. It was not possible to titrate by addition of successive increments of the macromolecule (Stone and Bradley, 1961) since some titrations would not be reproducible, possibly as a result of formation of anomalous complexes. In some instances, to permit full equilibration, the final scan was delayed five to ten minutes following the addition of the dye. The dye concentration in the mixture was normally 1×10^{-5} or 2×10^{-5}M, but in some of the experiments, where it was desirable to have extremely large excesses of polymer sites, lower concentrations of dye were used. At the normal concentrations, the absorption peak maximum of the aqueous dye was at 508 mμ, but at lower concentrations the peak shifted as much as 15 mμ toward longer wave lengths.

The stoichiometry of the reaction was determined from a plot of the decrease in absorbancy at 508 mμ against the P/D ratio the molar ratio of macromolecule anion sites to dye molecules. In most cases, the initial points formed a straight line with a definite break as sites began to exceed available dye molecules (see Figure 1) and only slight interpolation was required for accurate determination of the equivalence values. Similar results could be obtained, in some instances, by plotting the change in absorbance at the band maximum for the dye-macromolecule complex. In some cases, the plots were curvilinear, indicating a higher dissociation constant for the complex. In such cases, the equivalence values were estimated as the value at the intersection of the straight line tangential to the curve at P/D=0 with the straight line tangential to the curve at high P/D ratios.

2.2.2 EXTRACTION OF MINERALS

Four-gram samples of each mineral were weighed out and extracted for one hour with the aid of a mechanical shaker with 20 ml of a pretreating reagent (0.1M HCl, 0.1M EDTA, pH 4.5 and 0.1M $Na_4P_2O_7$, pH 4.5 were all used as pretreating agents). Some minerals were treated with only one reagent and other minerals tested with all the pretreating agents, a different mineral sample being used for each reagent.

The suspensions obtained were centrifuged for 10 minutes at 18,000 x g. The solutions were filtered through 0.22 μ Millipore filters and placed in dialysis bags. These samples were dialyzed exhaustively against distilled water on a rocking dialyzer for several days in order to remove the pretreating reagents before reaction with the dye.

The sedimented material from the pretreated samples was suspended in 20 ml of distilled water by use of a vortex mixer. The suspensions were centrifuged as before and the solutions dialyzed in the same manner against distilled water as the solutions obtained from the pretreatment step of the procedure. The residues from the water wash were suspended in 20 ml of 0.5N NaOH and extracted on the mechanical shaker for one hour. All solutions were filtered on .22 μ Millipore filters and were divided into two portions. One-half of each solution was dialyzed exhaustively against distilled water in a rocking dialyzer. The other portion of each of the solutions was dialyzed against 0.1N NaOH (dialysis was carried out in 4000 ml beakers with one change of 0.1N NaOH) for two days and then dialyzed against distilled water for several days in the rocking dialyzer.

The spectra of extract-dye samples were determined with a recording spectrophotometer as follows: 1 ml dye (6×10^{-5}M), 1 ml .01M Tris buffer at pH 7.5 and 1 ml of mineral extract.

2.2.3 EXTRACTION OF INORGANIC COMPOUNDS

Four grams of each compound were mixed with 20 ml of 0.1N HCl and allowed to mix for an hour with continual stirring. The solutions were decanted (with centrifugation if required) and water added to all the samples. One hour later the water wash was decanted and 0.5N sodium hydroxide was added to the samples.

The arsenite and persulfate solutions were filtered through 0.22 μ millipore filters while the Fe_2O_3 was filtered with the use of 0.8 μ millipore filter. The solutions were divided into two parts, one portion being dialyzed against distilled water and the other against 0.1N NaOH. Alkali or water was changed after 28 hours and dialysis continued for an additional 36 hours. All solutions were then dialyzed exhaustively against distilled water by use of a rocking dialyzer.

In the case of the polysulfide, a somewhat different procedure was utilized. A saturated solution of the Na_2S was prepared (~20 gms/100 ml). An excess of sulfur was added and the mixture allowed to shake for one hour in a rotary shaker, after which the solution was filtered. Four ml of the polysulfide solution was added to 20 ml of 0.1N HCl, but since the solution was still quite alkaline, concentrated HCl was added to acidify. At pH 2.1 sulfur separated as a light flocculent material floating on top of the solution. After filtration the precipitated material was suspended in 0.5N NaOH for one hour and then filtered again. The filtrate was divided into two portions and dialyzed in the same manner against water and alkali as for the other inorganic compounds tested.

2.2.4 SOIL EXTRACTION METHOD

Weighed soil samples were extracted with a pretreating agent (20%, w/v) by shaking for one hour on a mechanical shaker. Several pretreating agents, such as water, 0.1M HCl, 0.25 M lactic acid (pH 4.3), 0.1M EDTA (pH 4.5) and 0.1M $Na_4P_2O_7$ (pH 4.5), were tested in the various experiments. However, when 0.3M HF-0.3M HCl was used for pretreatment, the temperature was reduced to 4°C and a shaking period of 10 min. used. The suspensions were centrifuged at 18,000 x g for 20 minutes in the Lourdes Model A Betafuge. The soil residues were washed with distilled water or with 0.1M pyrophosphate, followed by water in the case of the HF-HCl or HCl pretreatment (mixing was carried out by use of a Vortex mixer), and centrifuged again.

The washed residues were resuspended in 0.5N sodium hydroxide (20% w/v) extracted for one hour at room temperature by shaking with a mechanical shaker and centrifuged (20,000 x g for 20 minutes). The supernatant solutions were placed in dialysis bags and dialyzed exhaustively against distilled water or against 0.1N sodium hydroxide for 2 days with a change of alkali every 24 hours followed by exhaustive water dialysis.

2.2.5 SILICATE TEST

The silica determinations were made by the procedure of Furman (1962, pp.960-963). Initially, a rough standard silicate solution which contained 5.0 gms of sodium metasilicate in 200 ml of distilled water was used for the study of the comparative removal of silicate by dialysis. However, for subsequent quantitative studies the silica standard was prepared as follows: Enough $SiO_2.n\ H_2O$ was added to a tared crucible to give an approximate final concentration of 0.1 mg Si/ml. The platinum crucible containing the silicic acid was brought to constant weight by alternate periods of heating (in the flame of a Meker burner) and cooling (in a vacuum desiccator). The pure anhydrous silica (0.1900 gm) was fused with 2 gms of Na_2CO_3 by heating at slightly above the fusion temperature for about 15 minutes. After cooling the melt was dissolved in warm water and transferred quantitatively to a 1000 ml volumetric and diluted to volume (.0888 mg Si/ml).

2.2.6 DIALYSIS SYSTEM

Water dialysis of many of the soil and mineral extracts was carried out by use of a closed dialysis system. This consisted of a rocking dialyzer to hold the samples contained in the dialysis tubing, a circulating pump and a mixed bed ion exchange column (Bantam replacement cartridge of Barnsted Still & Sterilizer Co.). Water was cycled through the system continuously.

Water dialysis was also accomplished by placing the sample (contained in dialysis tubing) in 4 liter beakers or by running distilled water continuously through a rocking dialyzer. Alkaline dialysis of soil extracts was carried out in 4 liter filter flasks adapted so nitrogen could be bubbled through the dialysis solution. Alkaline dialysis solutions (0.1N NaOH) were made up from boiled water by addition of 50 percent sodium hydroxide solution.

2.2.7 INFRARED ANALYSIS

Infrared spectra were recorded on KBr pelleted samples. Six hundred mg of KBr, 5 ml of glass distilled water and less than 0.5 mg organic carbon of the soil extract or the methylation or acetylation product were mixed, the water removed by freeze drying and subsequent storage overnight in a vacuum desiccator at 50°C. The dried samples were transferred in a dry glove bag to a KBr disc press which was subsequently evacuated and pressed at 10 tons total pressure for 20 minutes. Infrared spectra were recorded on a Perkin-Elmer Model 221 Infrared Spectrophotometer. Controls consisted of KBr to which distilled water was added and which was then subjected to the same procedure.

2.2.8 METHYLATION OF HUMIC ACID

Fifty mg of dry humic acid were placed in 5 ml of methanol and mixed with 1.5 to 2.0 ml of boron trifluoride etherate ($BF_3O(C_2H_5)_2$). The solution was sealed and placed in a warm water bath for 15 minutes. Water, (20 ml), was added to the solution and the solution neutralized. The methanol was removed by flash evaporation. This was accomplished by reducing the volume to one half, adding water to bring the solution up to volume and again flash evaporating the solution to half the initial volume. The solution was then dialyzed against distilled water for two days. The dialyzed product was employed for titration, infrared and dye studies.

2.2.9 ACETYLATION OF HUMIC ACID

To 20 mg of freeze-dried humic acid was added 5 ml of dry tetrahydrofuran and 5 ml of acetyl chloride. The mixture was placed in a water bath at 65°C and heated for 24 hours with occasionally swirling. The acetyl chloride and tetrahydrofuran were removed under vacuum at 30°C (solution not taken to dryness). Water (20 ml) was added and solution neutralized and lyophilized. The lyophilized material was taken up in 0.1N NaOH and dialyzed against distilled water for two days. The dialyzed material was employed in subsequent studies.

SECTION 3

RESULTS AND DISCUSSION

3.1 STOICHIOMETRY AND MECHANISM OF THE COMPLEXING REACTION*

The metachromatic color changes of dyes, which result from interaction of the dye with ionic macromolecules, have long been of interest in histochemistry. The mechanisms for these metachromatic reactions have been under study for three decades, but much confusion still remains as to the nature of the reactions. Recent hypotheses, based on dye-dye interactions upon the polymeric matrix (Bradley & Wolf, 1959; Wolf & Bradley, 1960; Stone & Bradley, 1961) seem to account satisfactorily for the relatively simple reactions of some dyes such as acridine orange. However, a dye such as 4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine bromide (DBTC)** reacts with polymeric anions to form at least five different and discrete complex states (Kay, et al., 1964a and 1964b). Such a complicated series of reactions has always been difficult to accommodate under the simple aggregation theories previously advanced (Bradley & Wolf, 1959; Wolf & Bradley, 1960; Scheibe, 1938; Scheibe, 1937; Scheibe & Zanker, 1958) and the studies presented below now demonstrate, unequivocally, the inadequacy of some of these theories as applied to the reactions of DBTC.

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* This section is the text of a paper accepted for publication in the Journal of Physical Chemistry.

** Abbreviations used in this section include: DBTC, 4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine; P, polymer anion site; D, dye molecule; RNase, ribonuclease; DNA, deoxyribonucleic acid.

In particular, the concept of the complex which creates an intense, narrow absorption band (J-band) at a longer wave length than that of the monomeric dye has had to be revised on the basis of these studies. This J-band was previously thought to arise from interaction of dye molecules in a large aggregate of the dye, either as a nematic crystal or on a polymer matrix or micellar structure (Kay, et al., 1964a; Kay, et al., 1964b; Scheibe, 1957; Scheibe & Zanker, 1958; Sheppard, 1942.) It now appears the J-band formed by the reactions of the dye with polymers is more likely to be due to reaction of individual dye molecules at isolated sites. This is more coincident with the original concept of Jelley (Jelley, 1936; Jelley, 1937), for whom this band was named, since he also considered it to be a molecular spectrum even in the nematic crystals.

In addition, it appears desirable to assume that the spectra of some of the other complex states may be caused by specific interaction of the dye with the polymer rather than by dye-dye interactions between adjacent dye molecules on the polymer. The more vital role of the polymer in the formation of the complexes may be further supported by the discovery that some of the dye-polymer complexes are almost completely independent of pH, suggesting that their formation is dependent upon forces other than the simple attraction of the negative anion site for the cationic dye.

Upon titration of the dye with a polyanion molecule, at constant dye concentration, the absorption spectrum of the reaction mixture progressively changes with increasing polymer anion site to dye molecule ratio until an equivalence point (usually at polymer anion site to dye molecule ratio of 1) is reached. After this point, little or no further change occurs, except in a few instances where extremely high ratios induce shifts toward new bands. As an example, the titration curve for the dye reaction with polyadenylic acid is shown in Figure 1. Titration with different macromolecules results in a number of distinct, stable spectral bands, each apparently characteristic of a certain type of complex. The various spectral bands obtained with the complexes are illustrated in Figure 2. Sheppard, 1942, designated several spectral bands of free dye as α, β, and γ in order of decreasing wave length. The α-band is given by the monomeric form of the dye while the other bands occur with some degree of dye aggregation. These designations have been retained for the free dye bands at 575 mμ(α), 535 mμ(β), and 500-510 mμ (γ) and for the corresponding bands of dye-polymer complexes. Reference to the band at 650 mμ as a J-band also follows Sheppard's terminology for the complex state. The sharp, intense band in the region of 470 mμ, arising under special circumstances, does not correspond to previously described bands, nor to known states of the free dye and has been termed, for convenience, the S-band (short wave length band). A seemingly discrete state with an absorption band at 550 mμ is frequently found. Although the manner of transition from the aqueous dye to the complex producing the 550 mμ peak is characteristic of a change to a unique energy state, several pieces of experimental evidence suggest that

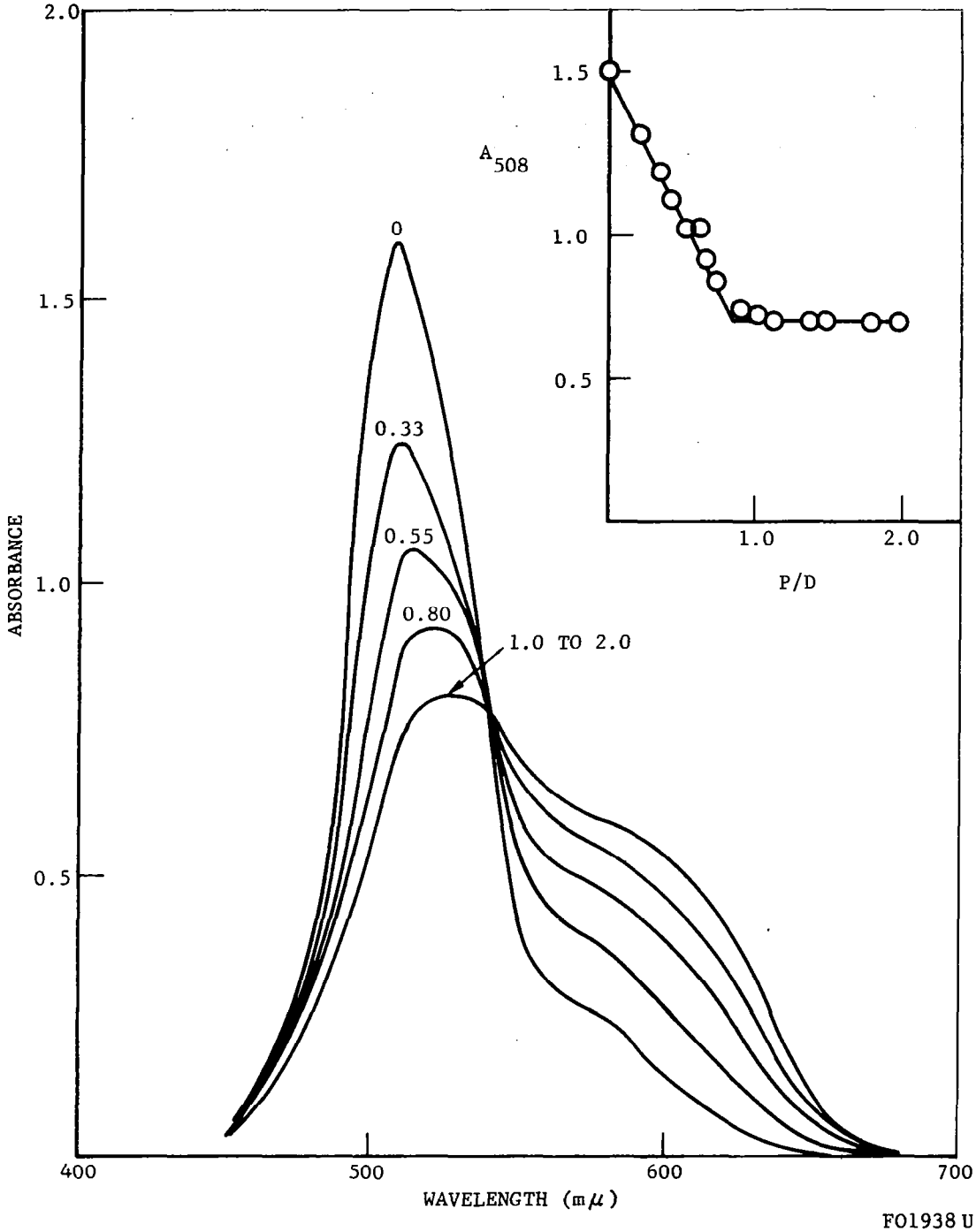

FIGURE 1. TITRATION OF DBTC WITH POLYADENYLIC ACID

DBTC at 2 x 10^{-5}M. Titration procedure as in text. Buffered at pH 6.4 with sodium cacodylate, 0.001 M. Numbers on the curves indicate polymer anion site to dye molecule ratio. (P/D) A_{508} = absorbance at 508 mμ.

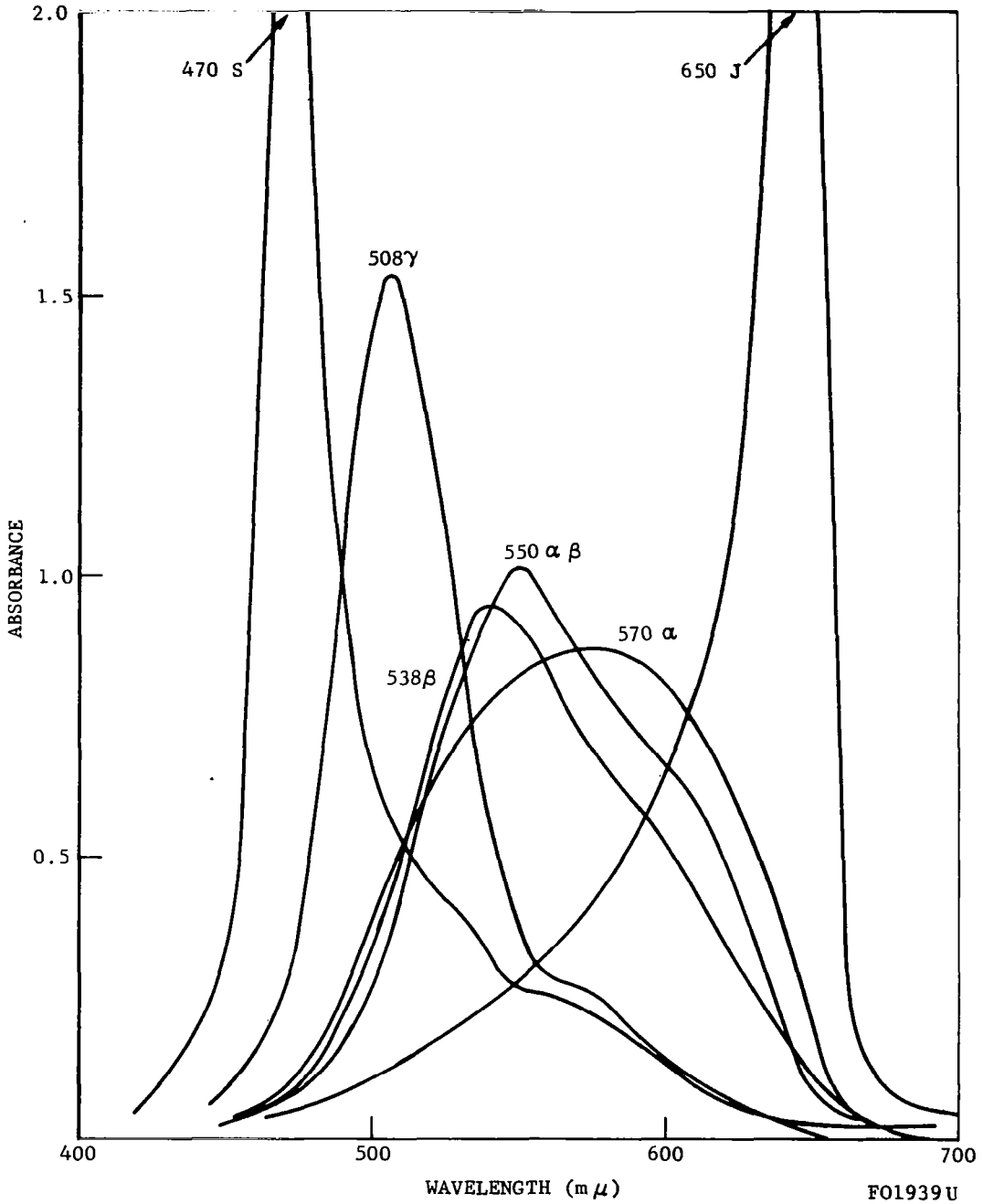

FIGURE 2. EXAMPLES OF THE DBTC - POLYANION COMPLEX SPECTRA

Spectra were obtained at stoichiometric reactions (except the 508 mμ peak) of dye and macromolecules. The 508 mμ curve (γ-state) is that for chymotrypsinogen but is essentially identical with that for the aqueous dye at 2.0×10^{-5}M. The 470 mμ curve, S-state, is for poly-L-lysine; the 538 mμ curve (β-state) is for polyaspartic acid; the 550 mμ curve (βa-state) is for polyglutamic acid; the 570 mμ curve (α-state) is for follicle stimulating hormone; the 650 mμ (J-state) is for pectin (10.8 percent methoxyl, fractionated sample).

this band may be either a hybrid or mixture of the α and β-bands. It will be designated here as the βa-band. A similar hybrid in the 600 mμ region will be called the αJ-band. Depending upon conditions (e.g., pH and temperature) and the particular polymer associated with the dye the bands may vary somewhat in position of maximum and in peak heights. However, they generally remain characteristic enough so that they can be considered to represent one of the complex states indicated above.

3.1.1 FORMATION OF THE STOICHIOMETRIC COMPLEXES

The α-state, the 570 mμ, α-band, is formed when the dye reacts with polygalacturonic acid, alginic acid, native DNA or follicle stimulating hormone (Table 1). The 570 mμ band is also found as a second band with a number of polymers which interact with the dye to form more than one band. The complex with polygalacturonic acid has a polymer anion site to dye molecule ratio near unity; that with alginic acid requires a ratio of two sites per dye molecule; the polymer anion site to dye molecule ratio for the DNA stoichiometric complex is also unity as illustrated in Figure 3. Although the free dye monomer and the α-complex both have their peak maxima at the same wave length, the α-complex band is much weaker and more diffuse.

3.1.2 THE β-STATE

This band is formed upon reaction with such diverse polymers as polyadenylate, polyaspartate, polyphosphate, heparin, and denatured DNA. The polymer anion site to dye molecule ratios required for complete reaction are close to unity for all of these except heparin which shows a polymer anion site to dye molecule ratio of 2.6 at full titration. Thus, all of the macromolecules which react with the dye to give a β-band are characterized by relatively close spacing of the anionic groups on the polymer. The titration with polyadenylate, Figure 1, is characteristic of this group.

3.1.3 THE γ-STATE

The γ-state in a complex is not always easy to demonstrate unequivocally. However, there are certain obvious cases which show that this state may occur because of reaction of the dye with a polymer. Thus, as shown in Table I, addition of myoglobin, bovine albumin, human albumin, hemoglobin or pepsin to the dye causes the peak to shift slightly to shorter wave lengths. In addition, a number of proteins, which do not cause a wave length shift, do create a drop in the absorbance of the band with a slight broadening of the peak. It can also be demonstrated, in several of these cases, that the dye is more stable to heat, light and extremes of pH, than the free dye, which indicates that there is an interaction between the dye and the polymer. For example, at pH 2 the dye alone changes rapidly to a pale yellow color, but in the presence of polyglutamate the color remains stable, while the maximum shifts slightly to 502 mμ. However, many of the

TABLE I

SUMMARY OF COMPLEXING REACTIONS OF DBTC
WITH VARIOUS MACROMOLECULES

Macromolecule	Type of Complex[a]	γ max mμ	P/D[b]	Polymer Equiv. Weight[c]	Conditions
α-group					
DNA, native	α	560-572	0.96		pH 7.0
Polygalacturonic acid	α	565	0.80		pH 7.0
Alginic acid	α	568	2.10		pH 8.2
Heparin, desulfated	α	565			pH 8.6
Follicle Stim. Hormone	α	570		9000	pH 7.2
β-group					
Polyadenylic acid	β	528-530	0.98		pH 5-9
Polyaspartic acid	β	535	1.18		
DNA, denatured	β	535	1.04		
Polymetaphosphate	β	535-538	1.24		pH 1.5-10
Heparin	β	535	2.6		pH 7.6
γ-group					
Albumin, bovine	γ	490-500			pH 8
Albumin, human	γ → J[d]	500→500+650[d]			
Hemoglobin	γ	495-505		1670	pH 8.6
Pepsin	γ → J	500→500+660[d]		<3500	
Myoglobin	γ → J	490→490+650[d]	(<4/10)	<3500	pH 7
Pepsinogen	γ → J	508→508+650[d]			
Trypsin	γ → J	508→508+630[d]			
Chymotrypsinogen	γ	508			pH 7.0
	γ → α	508→570	(5/13)	4500	pH 8.6
Polyglutamic acid	γ	502-510			pH <4

TABLE I., Cont'd

Macromolecule					
βa-group					
Polyglutamic acid	βa	550	0.92		pH >4
Polyuridylic acid	βa	545-560	1.06		pH 8
Chondroitin Sulfate	βa	555	0.98		
Alginic acid	βa	552	1.04		
Alginic acid, 2/3 methyl ester	βa	552	0.25		pH 8
Glycoprotein	βa	548		1000	
Heparin, desulfated	βa	552	1.0		pH 7-9
	αJ	605			pH 4.8
J-group					
Pectin, 10.8 or 11.3% Methoxyl (67% ester)	J	640	1.08, 0.98		pH 4-10
Pectin, 9.4% Methoxyl (56% ester)	J	640	1.02		pH 4.8
Pectin, 7.0% Methoxyl (41% ester)	J + α	615	1.2		pH 4-9
Pectin, 4.3% Methoxyl (24% ester)	J + α	613			
Pectin, 13.3% Methoxyl	J, α, γ	640,570,525	5.0		
Hyaluronic acid	J	635	2.28		pH 2-10
Ribonuclease, native	J	650	(7.3)	4500	pH >10
Ribonuclease, oxidized	J	650	(16/6)	3000	pH 5-10
Protamine Sulfate	J, α, γ	655,575,525		560	pH >10
Glutamyl-glycine, thermal copolymer	J	650		8000	pH 7.0
Gelatin	J	650		6700	pH 6.8
For other proteins in J-group, see Kay, 1963.					
S-group					
Poly-L-lysine	S	470	40		pH >10
Polymetaphosphate + $ZnCl_2$	S, β	475,535			Large excess of sites

TABLE I, Cont'd

a. Designation of complex states follow the terminology in the text and as shown in Figure 2.

b. P/D is the experimental ratio of macromolecule anion sites to dye molecules required for the stoichiometric reaction to produce the indicated complex. Determined by the methods outlined in the text. The fractions in parentheses indicate the apparent ratios of titratable anion sites of proteins to dye in the complexes.

c. Polymer equivalent weight is the weight of the macromolecule required for reaction with one gram molecule of dye. This is used in place of P/D for systems where the number of available sites may not be well defined.

d. Arrow indicates a time dependent transition from the initial γ-complex to the indicated final complex system.

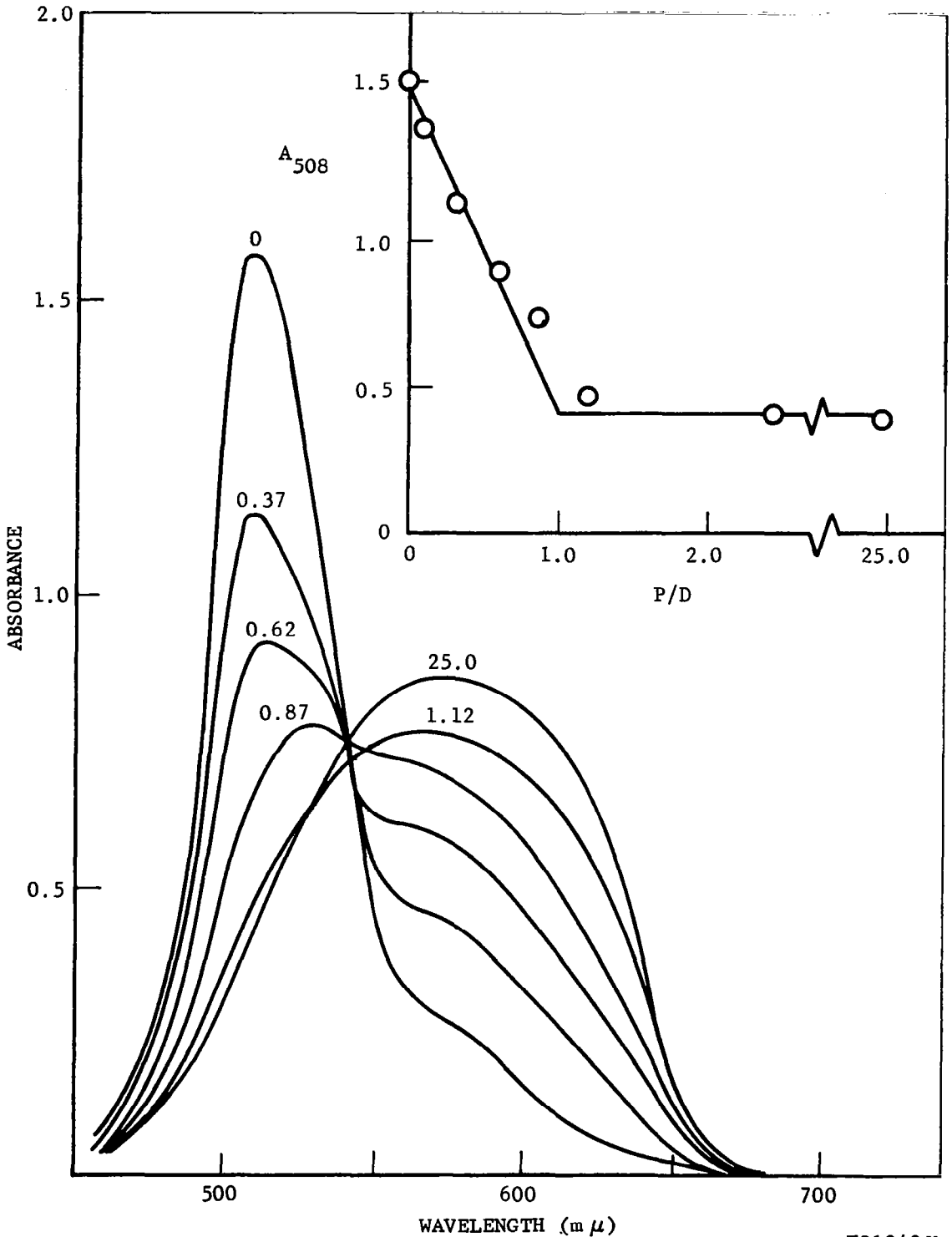

FIGURE 3. TITRATION OF DBTC WITH NATIVE DNA

DBTC at 2 x 10^{-5}M. Titration procedure as in text. DNA solution, 0.025% in DNA and 10^{-6}M in $MgCl_2$ was used in this titration. The reactions were buffered at pH 7.0 with 0.005 M cacodylate buffer. Numbers on the curves indicate P/D (polymer anion site/dye molecules) ratios.

γ-state complexes are apparently not entirely stable and a slower reaction ensues, creating a shift to one of the more stable systems, as indicated in the data in Table I. The βa-state is formed upon reaction of the dye with polyuridylic acid, chondroitin sulfate, polyglutamic acid, alginic acid (at polymer anion site to dye molecule ratio equal to one), methyl alginate and glycoprotein. The titration with polyuridylic acid (Figure 4) illustrates the formation of the βa-band. However, titrations to higher polymer anion site to dye molecule ratios cause opposing changes in two of the βa-complexes. An increase of the polymer anion site to dye molecule ratio equal to two with alginic acid causes a shift to the α-band, while polyuridylate ratios in excess of 200 cause shifts toward the β-band. The characteristic βa-band may be reproduced by the reaction of some samples of partly denatured DNA. This band is obviously a mixture of the α-band of native and β-band of denatured DNA. Thus, the existing evidence seems to suggest that the 550 mμ band is a hybrid or mixture of the α and β-states.

3.1.4 THE J-STATE

The unique spectrum of the J-state is found, unequivocally, for such materials as pectin (degree of methylation more than 66% or 10.8% methoxyl content), hyaluronic acid, a number of proteins (oxidized ribonuclease will be the primary example) protamine (at higher pH) and a thermal polymer of glutamic acid and glycine. Pectin (Figure 5) and hyaluronic acid have been the only relatively uncomplicated molecules upon which to base stoichiometric studies. Pectins which are less than two-thirds methylated (10.8% methoxyl) also show an α-component (Table 1), but the reaction is complete at a polymer anion site to dye molecule ratio of unity regardless of whether a pure J or α-component (Table 1), but the reaction is complete at a polymer anion site to dye molecule ratio of unity regardless of whether a pure J or α plus J-state is exhibited. However, in the hyaluronic acid reaction, the dye apparently occupies only alternate sites (polymer anion site to dye molecule ratio equal to 2.3). The proteins have a reaction equivalent weight varying from about 2000 to 8000 per dye molecule (grams protein per gram mole of dye) which varies rather widely with pH in some cases and with other environmental factors. It is difficult to determine, for proteins, the actual number of available anion sites, since many of the carboxyl groups may be masked. Native ribonuclease reacts with the dye to give a J-band only at a pH above its isoionic point. The native RNase contains 8 carboxyl groups and the titration requires only 3 to 5 dye molecules per molecule of RNase. The oxidized RNase, with eight cysteic acid residues added to the original carboxyls, requires between 5 and 6 dye molecules per molecule of RNase.

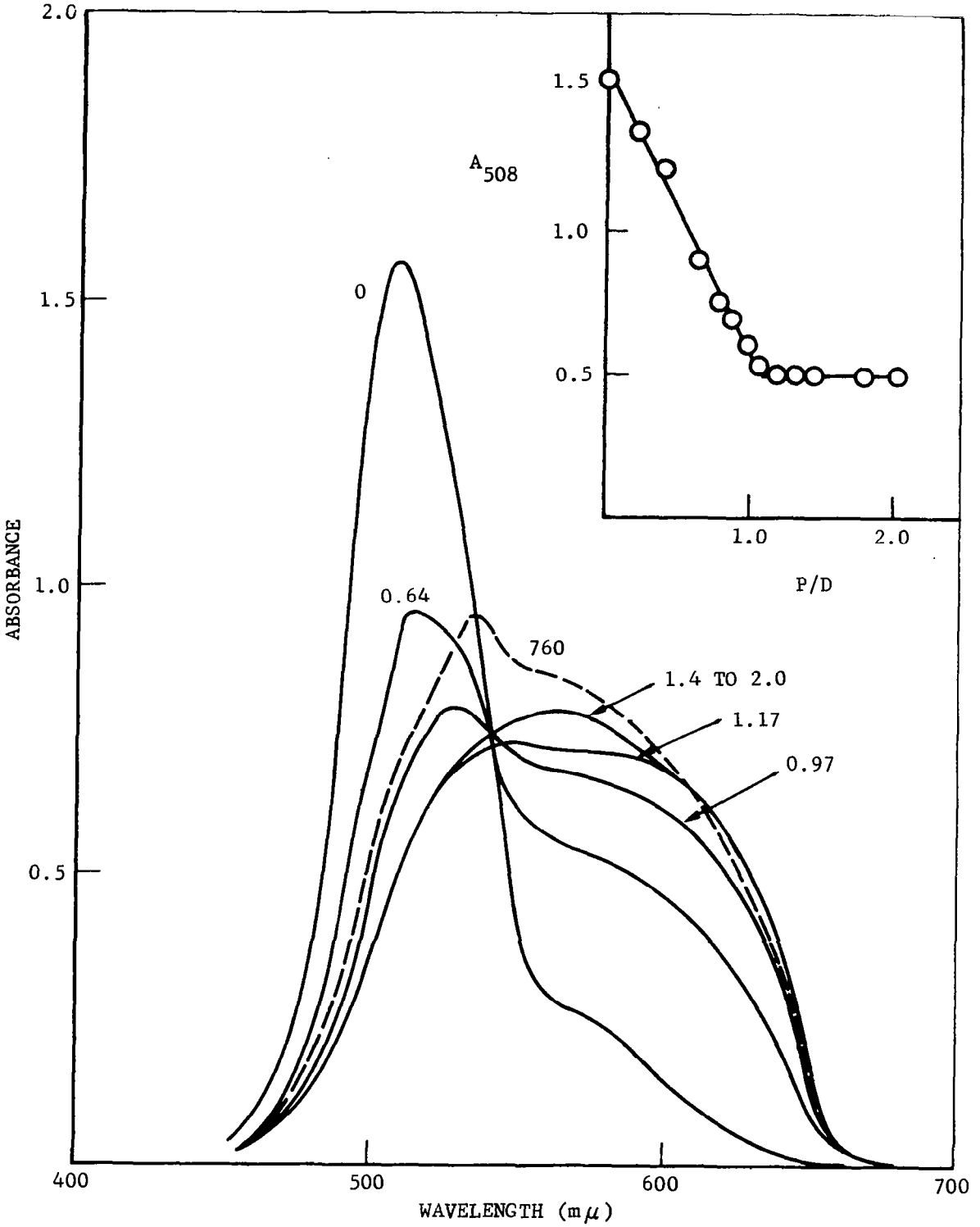

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FIGURE 4. TITRATION OF DBTC WITH POLYURIDYLATE

DBTC at 2 x 10^{-5}M, buffered at pH 6.4 with cacodylate, 0.016 M. Numbers on curves indicate P/D (polymer anion site/dye molecule) ratios.

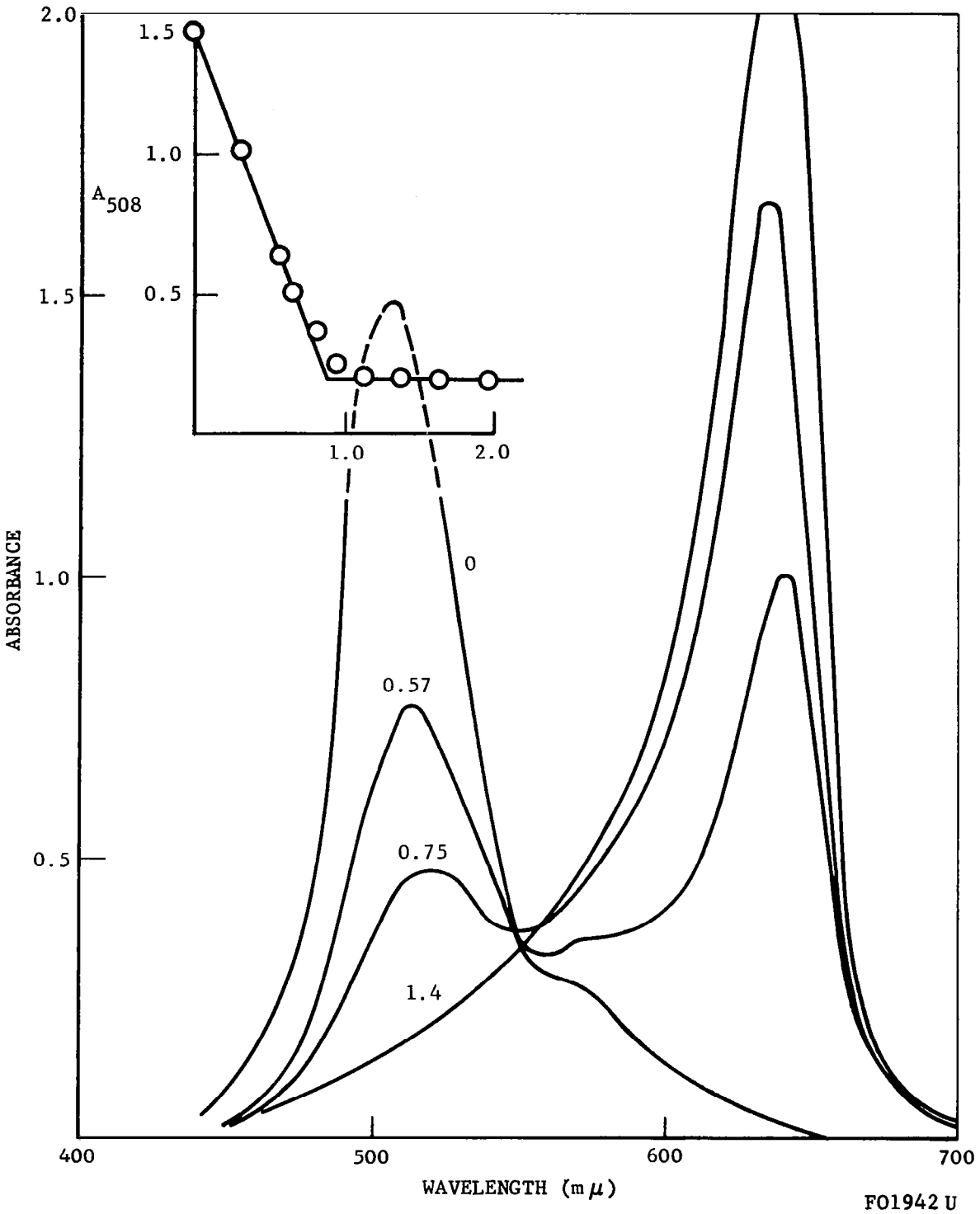

FIGURE 5. TITRATION OF DBTC WITH PECTIN (10.8 PERCENT METHOXYL)

DBTC at 2 x 10^{-5}M; solutions buffered at pH 4 with succinate, 0.001 M. Numbers on curves indicate P/D (polymer anion site/dye molecule) ratios.

3.1.5 THE S-STATE

The S-complex is an interesting, but poorly defined state. It is found upon titration of the dye with polylysine hydrobromide, at a pH greater than 10, and as a secondary band in a number of other instances. It can also be produced with polymers which normally form other bands through the influence of specific cations at high polymer anion site to dye molecule ratios, e.g., mixtures of $ZnCl_2$, at 10^{-3}M, with polymetaphosphates. It also appears upon reaction with certain anions, such as borate, at high pH. The extent of the polylysine reaction is also dependent upon pH, with a greater reaction occurring as the pH increases beyond the initial reaction point. This reaction is also inefficient in regard to the number of polymer sites required. Thus, about 40 polylysine residues are needed for each dye to obtain complete reaction at pH 11, and many more at pH 10.5. This suggests that the 470 mμ band may arise as a consequence of a complex between the cationic site on the polymer, hydroxyl ion (or other ion) and the dye.

3.1.6 CHANGES IN SPECTRA INDUCED BY HIGH ANION SITE TO DYE RATIOS

As the polymer anion site to dye molecule ratio is increased from zero to unity in the reaction of acridine orange with polymers, this dye demonstrates a hypsochromic shift to the β-band. As the polymer anion site to dye molecule ratio is still further increased, there is generally a tendency for the absorption spectrum to return to that of the monomeric dye, namely, the α-band. It has been proposed (Bradley & Wolf, 1959 and Wolf & Bradley, 1960) that the β-band, for acridine orange, is due to dye-dye interaction in a closely packed sequence of dye molecules on the polymeric matrix. As the ratio of sites to dye molecules becomes increasingly large, the dye molecules may then become randomly distributed at widely spaced, non-interacting sites, giving rise to the spectral characteristics of the monomeric dye. Since the number of excess sites required to promote this latter shift varies widely for different polymers, Bradley introduced the concept of the "stacking coefficient", which is the measure of the apparent tendency for dye molecules in the presence of excess sites to occupy sites adjacent to other dye molecules in preference to other available sites.

Previously (Kay, et al. 1964a and 1964b), it has also been assumed that the α-complex of DBTC with polymers is a monomer equivalent, while the β, βa, and J-complexes all involved some degree of dye-dye interaction. One would then expect that very high ratios of sites to DBTC would also cause some degree of shifting toward the α-complex spectrum in analogy to the acridine orange reactions. This hypothesis has been tested in a number of instances with DBTC (Table 2).

TABLE II

CHARACTERISTICS OF THE DBTC-MACROMOLECULE COMPLEXES

AT HIGH P/D[a] RATIOS

Macromolecule[b]	P/D[a]	λ max mμ	pH
Polyadenylate (530)	10	530	7.0
	250	530	7.0
	708	533	7.0
Polyuridylate (550)	28	550	7.0
	280	540	7.0
	765	535	7.0
Polyaspartate (535)	11	536	8.6
	20	538	7.0
	50	538	7.0
Polyglutamate (550)	10	550	8.6
	32	547	7.0
	480	547	7.0
Deoxyribonucleic acid (570)	11.5	570	7
	57	573	7
	115	574	7
	230	575	7
Pectin, 10.8% methoxyl (645)	11	655	5.0
	500	635,595	5
	1580	555,635	5
Pectin, 7.0% methoxyl (590)	10	570	5.0
	330	595,640	5
Polygalacturonic acid (565)	26	570	7.0
	53	560	7.0

TABLE II, Cont'd

Polymetaphosphate (535)	20	535	
	80	540	
	825	542	
	1650	543	
	16,500	551	
	31,500	555	
	80,000	555	
	225,000	552	
Alginic acid	1.0	552	8.6
	2.1	575	8.6
	17	570	8.6
	34	564	8.6
	100	560	8.6

a. P/D is the experimental ratio of macromolecule anion sites to dye molecules required for the stoichiometric reaction to produce the indicated complex. Determined by the methods outlined in the text.

b. Figures in parentheses indicate λ max of the normal stoichiometric complex.

Among the reactions tested, one, alginic acid, shifts from the β-complex to the α-complex at a low ratio (2:1). But at still higher ratios (34:1) there is evidence for a shift back toward the shorter wave lengths and the spectra show the presence of a γ-component. The polyphosphate band also shifts in the expected direction, from a pure β-band at 535 mμ, to one with a significant α-component having a maximum at 556 mμ. However, this only occurs at very high ratios, a ratio of 31,000 being required for the maximum shift. Further increases in polymer anion site to dye molecule ratios produce no further changes.

Polyuridylic acid, at high ratios, shows an inverse shift, toward the β-complex (see Figure 4, dashed curve). Pectin spectra also vary with polymer anion site to dye molecule ratios. Pectins of relatively low methoxyl content show definite increases in a J-band component at high ratios (counter to the hypothesis), but the higher methoxyl samples (10.8 percent methoxyl), which demonstrate pure J-bands at stoichiometric levels, acquire an α-component at polymer anion site to dye molecule ratios varying from 58 to 1580. None of the other complexing reactions tested showed significant variations in the ranges tested. Thus, of the four systems which show changes with increasing polymer anion site to dye molecule ratios, only alginic acid and polymetaphosphate unequivocally shift in the direction which might be expected for a transition from a close-packed complex, with dye-dye interaction, to more widely spaced, non-interacting complexes. In contrast, polyuridylate and low methoxyl pectin, produce changes in opposition to those expected.

These studies demonstrate that the DBTC complexes do not follow the simple behavior of those described for acridine orange, making it difficult to retain the simple dye-dye interaction hypothesis for these complexes.

3.1.7 EFFECTS OF OTHER FACTORS ON THE COMPLEX

Anion Group

The anionic sites of the polyanions examined here include carboxyl, phosphate, sulfate and cysteic acid groups. The carboxyl groups apparently may participate directly in forming any of the spectral species since representatives may be found in the J-band (pectins, hyaluronic acid), the α-complex (pectic and alginic acids), the βa-complex (polyglutamate) and the β-complex (polyaspartate). The carboxyl groups of the proteins are probably also responsible for the formation of the γ-complexes. The S-band is also associated with polymers containing carboxyl groups, but only as a secondary band.

No polymer containing only phosphate anionic sites has been found specifically to form either the J-complex or the S-complex, but each of the other complexes form with phosphate sites. These include DNA for the α, polyadenylate for the β, and polyuridylate for the βa complexes.

No complexes have yet been tested which rely only upon the sulfate or related groups so it is not possible to establish specificity for them. However, since the two sulfated representatives, chondroitin sulfate and heparin, show the βa and the α-bands, it may be assumed that these states are not excluded by sulfate.

The S-complex, as noted previously, arises under special circumstances and appears to be associated with high pH. However, a small secondary band occasionally arises in the same region under some conditions (either excess dye or excess sites) with other polymers.

Thus, the nature of the anion site seems to have little direct influence upon the type of complex formed except that the carboxyl is more readily associated than the other groups with the J-complex. An exception to this has arisen in recent, uncompleted studies where it has been found that polysaccharide-borate complexes (starch and borate) give a J-band reaction with the dye.

Anion Site Spacing

Anionic sites upon a polymeric matrix are tremendously more effective than the corresponding simple anions in creating the specific complexes with DBTC (Kay, et al., 1964a & Kay, et al., 1964b). It is quite apparent, therefore, that this selective specificity must reside in the relation of the site-complex to the polymeric matrix or in the interaction between the adjacent site-complexes, or in a combination of both of these factors. It is worthwhile to examine some of the potential spacing relationships in the complexing systems.

The experiments with the pectins are most pertinent to the question of effect of spacing. Pectic acid (polygalacturonic acid), which has a carboxyl group on each uronide unit, reacts with DBTC to form an α-complex. Pectin, even with a methoxyl content as low as 4.3 percent (one out of four carboxyls methylated) shows a strong J-band in addition to the α-band upon reaction with DBTC. The J-component shows only a slight increase for a pectin with 7.0 percent methoxyl (about one out of three groups esterified). But, as the methoxyl content increases to 9.4 percent (56 percent ester) the α-component decreases rapdily as the J-band increases until, at 10.8 percent methoxyl (two out of three carboxyls esterified), a pure J-band is obtained in the DBTC reaction (Figure 6). Thus, as the average spacing between anionic groups in pectic acid and pectins is increased, there is a shift from the α-band to the J-band reaction. In the pectic acid, the anion groups are so closely spaced that there would be a high probability for interactions between adjacent dye molecules. Even if the conformations of the pectic acid made it impossible for adjacent dyes to interact (e.g., carboxyls on alternating sides of the polymer chain) the dye is large enough so that overlapping could still occur between dyes on alternate sites. On the other hand, as illustrated in Figure 7, anion sites for the

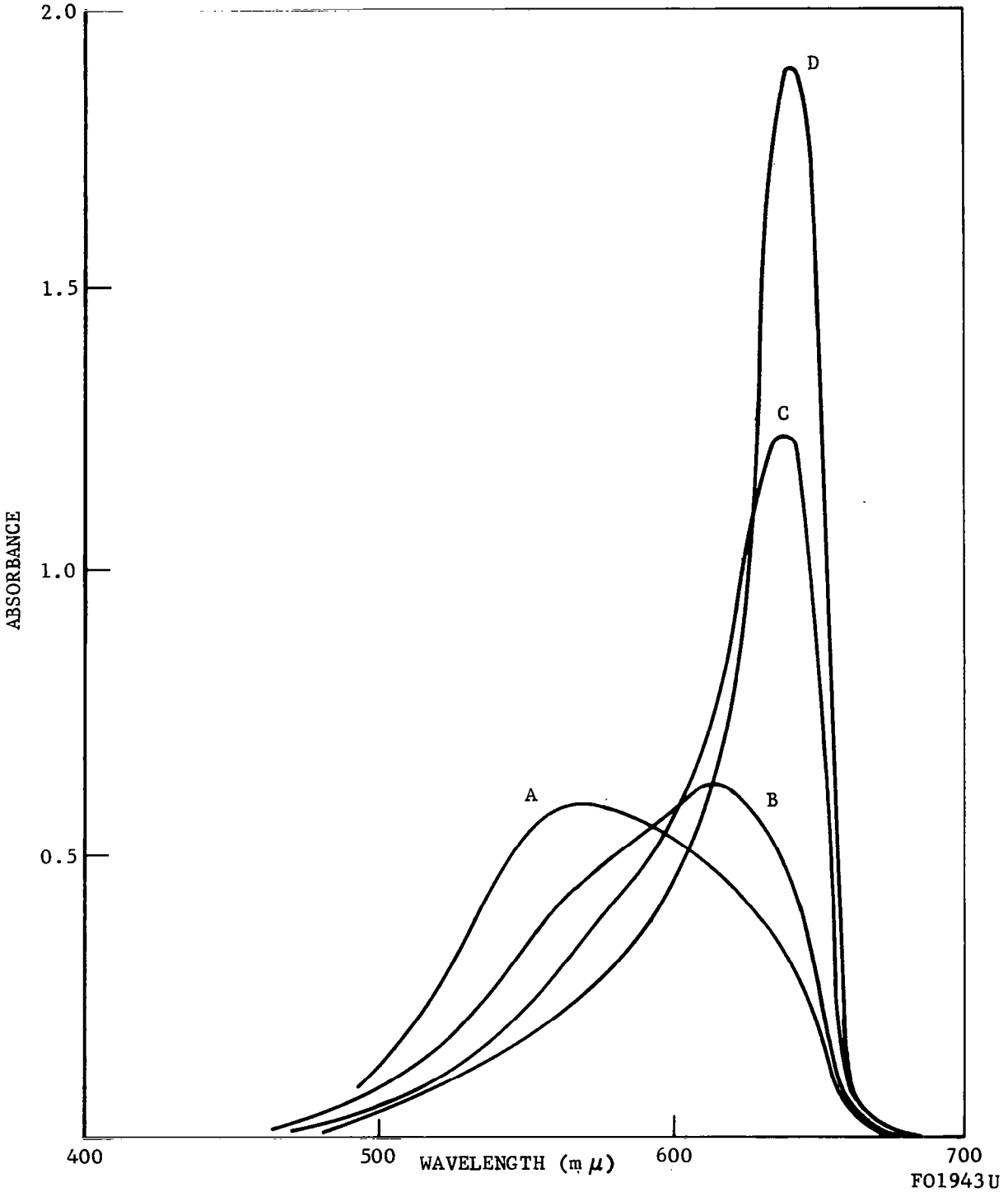

FIGURE 6. CHANGE OF DBTC-COMPLEX SPECTRA WITH INCREASING PECTIN METHYLATION

A - polygalacturonic acid (Sunkist sample, pH 7.0), P/D (polymer anion site/dye molecule) = 1.7; B - fractionated pectin, 4.3 and 7.0 percent methoxyl pH 4.0 (identical curves), P/D = 2.5 and 1.4 respectively; C - fractionated pectin, 9.4 percent methoxyl, pH 4.0, P/D = 1.5; D - fractionated pectin, 10.8 and 11.3 percent methoxyl, pH 5.0, P/D = 1.3.

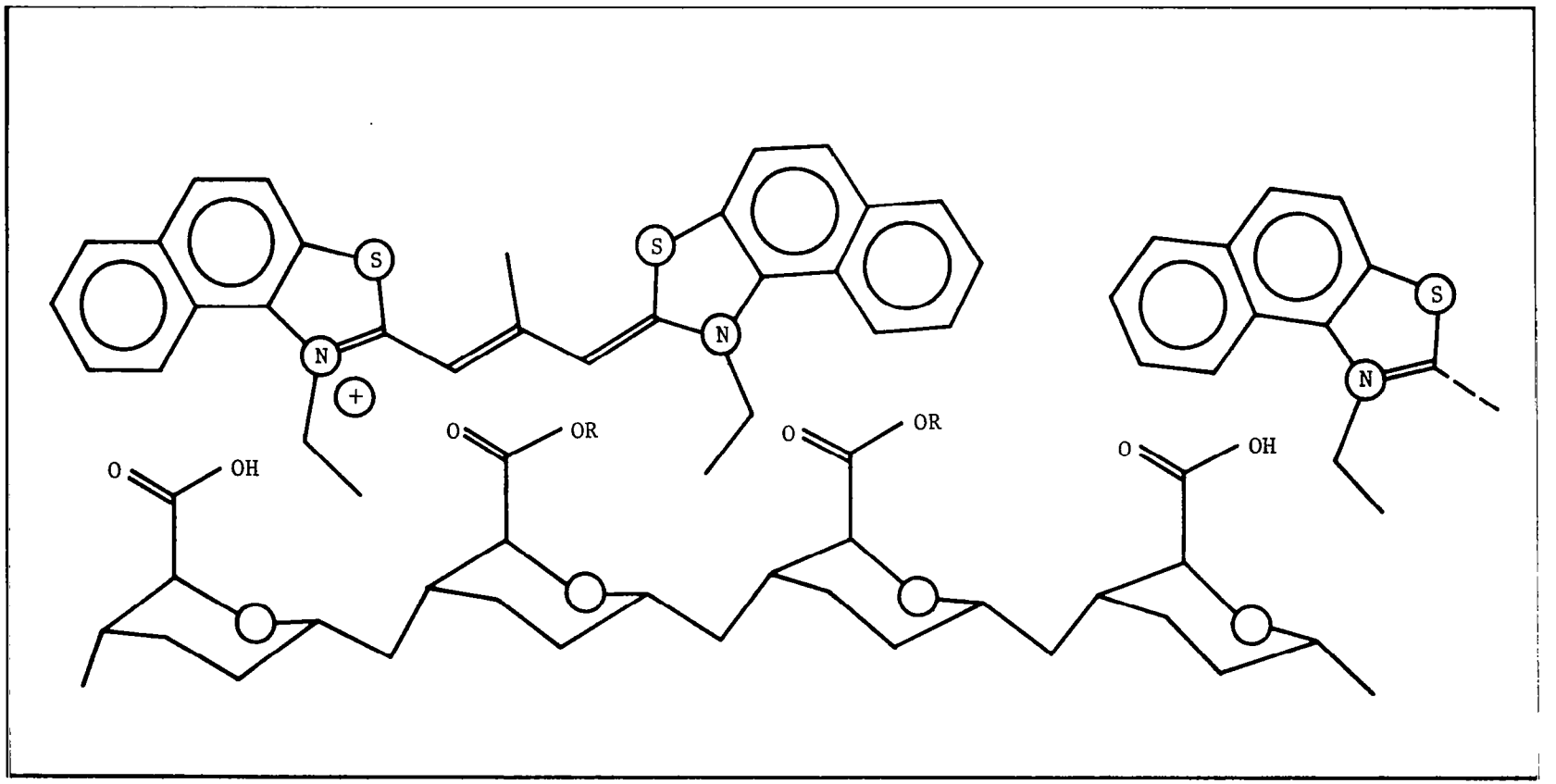

FIGURE 7. RELATION OF DBTC MOLECULAR LENGTH TO PECTIN ANION SITE SPACING

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This illustration only compares the length of the DBTC molecule with distance between anion sites on the pectin chain. No attempt has been made to indicate the actual spatial relations in this complex or the true conformation of either the dye or pectin molecules.

pectin having two-thirds of its carboxyls methylated are so widely spaced that interaction between dyes along the length of the polymer chain would be unlikely. Experiments to determine the effect of even greater spacing required chemical esterification of the pectin to obtain higher methoxyl contents. The chemically enriched samples, of 13 to 13.5 percent methoxyl, show extensive variations which depend upon the type of reaction used in esterification so these data cannot be applied to the question of spacing.

Hyaluronic acid (alternating glucuronic and acetyl glucosamine units) does not react in a one to one anion site to dye ratio, only one dye reacting for every 2.3 carboxyl groups. A strong J-band is also obtained for this polysaccharide, presumably as a result of the very wide spacing of the dyes on the polymer. These cases seem to contradict the concept that the J-band is due to an interaction of dyes in a large aggregate, while the α-band is formed when there is no dye-dye interaction in the complex.

Similar conclusions may be drawn from the ribonuclease data. A proposed structure and conformation of this protein (Avey, et al., 1962) would indicate that most of the anionic sites on the surface of the molecule are too widely spaced to permit interactions between dye molecules associated with them. Even allowing for errors in the conformation and secondary structure, a relatively wide spacing is suggested by the primary structure making it highly unlikely that the J-band found with ribonuclease can be due to interaction between a large number of dye molecules in a large dye aggregate as previously proposed.

Effect of pH

Most amphoteric polymers, such as proteins and nucleic acids, either fail to react with the dye or show a much-reduced reaction at a pH at or below their isoionic point (Table III and Kay, et al., 1964b). Thus, native RNase reacts only at a pH greater than 9 and shows a full reaction only at a pH of 10 or greater, while oxidized RNase, with a much lower isoionic point, reacts strongly at pH 4.0. RNase, which has been treated with formaldehyde to eliminate the effect of lysine groups proximal to the anionic sites or treated with nitrous acid to reduce the basicity of the histidine groups, reacts at a lower pH. A strong J-band is found for nitrous acid-treated (and dialyzed) oxidized RNase even at pH 3.0. Polyadenylate and polyuridylate also show some degree of sensitivity to low pH, but in each case it seems to reduce the intensity of the normal band with little shifting from the type of reaction observed. Polyglutamate shows a γ-complex at low pH in place of its βa-complex observed in neutral solutions. DNA acquires a β-component in addition to its α-band at low pH, but this is undoubtedly due to acid denaturation of the DNA.

TABLE III

EFFECT OF pH ON THE DBTC COMPLEXES

Complexing Macromolecule	pH	λ max[a] mμ	A max[b]	Buffering[c] Agent
J-band series				
Ribonuclease, native, (0.0022%)				
DBTC = 1 x 10^{-5}M	8.0	508	0.63	T
	8.6	512	0.53	T
		645	0.04	T
	9.1	512	0.52	O
		645s	0.13	
	9.4	525	0.32	O
		645	0.54	
	10.1	530s	0.13	O
		650	1.51	
	11.5	630	0.73	O
Ribonuclease, oxidized, 0.0022%				
DBTC = 1 x 10^{-5}M	3.4	523	0.35	S
		565	0.22	
	3.8	526	0.28	S
		580	0.33	
		653	0.59	
	4.2	530s	0.19	S
		650	1.25	
	5.0	650	1.46	S
	6.1	650	1.78	C
	7.0	650	1.57	C
	8.1	645	1.31	T
	8.9	642	1.15	O
	10.6	603	0.63	O
	11.1	593	0.61	O
Hyaluronic Acid, 0.002%				
DBTC = 2 x 10^{-5}M	2.0	650	>2	
	7.0	650	>2	
	8.8	650	>2	
Pectin (10.8% methoxyl), 0.00073%	2.6	645	0.23	O
	3.05	643	1.29	S
DBTC = 1 x 10^{-5}	8.4	642	1.69	T

TABLE III, Cont'd

Protamine, 0.007%	8.6	Dye curve		O
	9.9	525	0.35	O
DBTC = 1×10^{-5}		575	0.35	
		650	0.45	
α-band series				
DNA, native, 0.014% in 0.2 mM $MgCl_2$	2.0	538	1.17	O
		570	0.97	
DBTC = 2.7×10^{-5}	3.0	536	1.44	O
		567	1.23	
	4.0	568	1.45	O
	5.0	568	1.52	O
	6.5	568	1.61	O
	7.4	565	1.68	O
	8.8	568	1.59	O
	10.0	565	1.49	O
	11.5	572	1.26	O
Alginic Acid 0.0006%	2.0	642	0.59	
	2.5	625	0.83	
DBTC = 2×10^{-5}	3.1	610	0.97	
		570s	0.89	
	3.4	605	0.98	
		570	0.92	
	4.2	570	1.09	
	8.6	570	1.13	
	10.5	570	1.02	
Polygalacturonic acid 0.0022%	3.0	575	0.45	GG
	4.0	568	0.52	S
	7.0	652	0.52	C
DBTC = 1×10^{-5}	8.6	560	0.49	
β-band group				
Polymetaphosphate, 0.0067%	1.5	534	0.66	O
	3.5	536	0.94	O
DBTC = 2×10^{-5} M	4.9	538	0.96	O
	6.7	534	1.16	O
	8.2	534	1.12	O
	9.8	535	1.01	O

TABLE III, Cont'd

Polyadenylate	1.9	530ppt	0.49	O
	2.3	575	0.42	O
	3.4	520	0.84	
		570s	0.51	
		540	1.04	
	4.0	570	0.86	S
	8.2	530	0.89	T
	11.5	530	0.97	O
Heparin, native, 0.006%	2.6	530	0.55	O
	4.0	535	0.71	O
DBTC = 2×10^{-5} M	8.8	530	0.82	O
	11.3	533	0.84	O
<u>βa-band group</u>				
Polyglutamate, 0.0067%	2.0	502	0.77	O
	3.0	510	0.96	O
DBTC = 2×10^{-5}	5.0	540	0.89	S
	7.9	540	1.00	T
	8.8	550	0.99	T
	11.3	550	1.15	O
Polyuridylate, 0.0067%	6.3	560	0.79	O
DBTC = 1×10^{-5}	8.2	555	0.82	T
Heparin, desulfated, 0.0067%	4.8	610	0.99	
	9.3	555	0.90	
DBTC = 2×10^{-5}	10.5	553	0.93	
	11.0	552	0.90	

a. λ max indicates major peaks or shoulders (s) of the complex spectra.

b. Absorbancy at the indicated peak maxima with dye at indicated concentration.

C. Buffer symbols: O = no buffer, adjusted to indicated pH with acid or base before addition of the dye; T - Tris (Hydroxyl ethyl) amino methane; S = succinate; C = cacodylate; GG = glycyl-glycine. All buffers 0.001 to 0.003 M.

On the other hand, the polysaccharide and polyphosphate complexes are relatively independent of pH. For example, the hyaluronic acid complex is unaffected with variation of pH from 2 to 10; the J-band or α-band of pectins and the β-band with heparin show little change between pH 2.6 and 10; the β-band of the polyphosphate complex is stable from pH 1.5 to 10, except for changes in the intensity of the peak. Alginic acid below pH 4 shows an αJ-hybrid rather than the normal α-band, but this is probably due to precipitation of the free acid of the polysaccharide at low pH and consequent formation of micelles. Desulfated heparin also is affected by pH in that an αJ-complex is observed at a pH below 5 while the βa-complex is found above this pH. Again, this may be the result of a change in solubility of the polysaccharide.

3.1.8 DISCUSSION

In earlier papers on the metachromatic reactions of DBTC with polymers (Kay, et al., 1964a & Kay et al., 1964b), we assumed that the appearance of a specific spectral band in the complexing reaction indicated the presence of a specific state of dye aggregation. Thus, the α, β, γ, and J-bands were associated with progressively higher states of aggregation and polymerization of the dye on the surface of the polymers. These assumptions were then compatible with our knowledge of the behavior of the dye in free solutions and with the theories advanced by other authors for the metachromatic reactions of dyes. These hypotheses have all assumed that the different complex states were basically due to differences in degree or type of dye-dye interaction in the complex. The polyanion is presumed to play a passive part by providing a matrix with anion sites at intervals which would govern the distance of the dye molecules from one another. Thus, Bradley and co-workers (Bradley & Wolf, 1959; Wolf & Bradley, 1960; Stone & Bradley, 1961) for acridine orange, propose that the α-band is due to a monomeric species in both the complex and free dye, while the β and γ-bands of the free dye in solution are due to dye-dye interactions in aggregates (dimers or higher) and equivalent interactions between dyes on adjacent anion sites are responsible for these bands in complexes. With respect to the J-band, Jelley (Jelley, 1937) believed these bands to be due to molecular species; but his ideas have been superseded by those of Scheibe and Zanker (1958) and Sheppard (1942) whose theories require interaction between large numbers of dye molecules. Scheibe (1938) proposed that the J-bands resulted from overlap of the "electron clouds" of the conjugated systems in adjacent dye molecules which permitted formation of a new "electron cloud" common to the entire polymer--essentially a super-conjugated molecule. Scheibe's concept was criticized by Sheppard with the comment that the proposed complex would be "more metallic than metals". In its place, he offered a proposal that the J-band arises from the formation of cells of dye molecules having coupled dipoles, with water molecules entering into the complex to act as the coupling agent between the dyes. With a series of such coupled dipole cells, excitation energy could be propagated along the entire length of a polymer or thread consisting of stacked cells. Although the need for a high aggregate was not implicit in this concept, since the spectra would be similar for small as well as large

groups of cells, he assumed that the aggregates were actually responsible for the phenomenon. He also assumed that the J-band complex formed in reactions with polymeric substances, such as gelatin, was due to a similar aggregate formed at the surface of micelles of the polymer.

Our present data conflicts with some of these concepts, most particularly with the mechanism for the J-band formation. Considering the stoichiometry and spacing relations in the complexing reactions of DBTC with pectin it does not seem that the J-band can arise in complexes as a consequence of highly organized, large aggregates of dye molecules. Since optimal reactions are always obtained with a maximum of one dye molecule per anion site, any aggregate would have to stretch along the surface of the macromolecule with spacing controlled by the distance between anionic sites. If sites are sufficiently close, the interaction phenomena could account for the J-band reaction. Actually, the evidence suggests that there is a much greater probability for formation of the J-complex when sites are sufficiently separated so that there is little chance for interaction between adjacent dye molecules in the macromolecule complex. Thus, methylation of the anion groups of pectic acid, which would decrease the chance of formation of any long, surface aggregates of dye, causes a shift from the α-complex to the J-complex. A pure J-band is obtained coincident with a degree of esterification which would create an average spacing between anion sites (two ester groups for every free carboxyl) just slightly greater than the full length of the extended DBTC molecule. It is true that carboxyls on adjacent turns of a helix structure would be sufficiently closely spaced to permit dye-dye-interaction but such conformation has not been indicated for pectin. Reactions with ribonuclease reinforce the conclusion that the J-band may occur as the result of the interaction of individual DBTC molecules with isolated anion sites.

Similarly, the mechanism for the formation of the other complexes of DBTC must be modified to some degree. Although it has been attractive to assume that the α, β, and γ-bands are due to increasing degrees of aggregation on the macromolecular surface, under the influence of specific site spacings, the present data is not entirely consistent with this concept. Different spectra are obtained for complexes with macromolecules having the same anionic groups with similar spacing between sites, as in the case of the varying reactions for different polynucleotides. On the other hand, similar spectra may be found for the complexes arising from the reaction of DBTC with polymers having extremely variant spacings as evidenced by the β-band appearing with polymetaphosphate, polyaspartate and polyadenylate. Finally, the aggregation theory requires that a shift to a "monomeric" complex should occur at very high ratios of anion site to dye (Bradley & Wolf, 1959; Wolf & Bradley, 1960). Increasing the anion site to dye ratio during formation of DBTC complexes may cause no change at all or create shifts in either direction, toward or away from the supposed monomeric complex. Such behavior is very difficult to reconcile with a simple aggregation, dye-dye interaction hypothesis. Even with a very high

"stacking coefficient (Wolf & Bradley, 1960) (the measure of the tendency for a dye molecule to occupy sites adjacent to other dye molecules in preference to isolated sites) the reverse shifts cannot be explained except through the assumption of specific interactions between the site and the individual or aggregated dye molecules.

It seems that the reactions of DBTC with macromolecules might be better accommodated under a hypothesis which does not stress, exclusively, the dye-dye interactions in the complexes, but considers, also, the conformations of the dye and its interactions with the polymer site and adjacent groupings. In this case, specific bands would be obtained due to interactions of the dye with its site and/or neighboring dye molecules. These interactions would enforce certain conformation of the dye molecule and reinforce (increase transition probabilities) specific vibrational and electronic combinations in the dye molecule. This mechanism would be compatible with the concepts of Sheppard, who stated, with respect to the formation of the β and γ-bands of the free cyanine dye, "--we consider that the doublet or dimer formation accounts for the spectral deviation. But this is not because β or γ-bands of higher frequency are new bands peculiar to the 'polymers' but because the doublet formation enhances the transition probabilities of bands produced in the molecule by coupling of the electronic transitions with vibrations." We are in agreement that dye interactions in the polymeric complexes create the observed spectral shifts, but it should not be assumed that it is only coupling between dye molecules which induces these effects; they may be similarly induced by a change in conformation of the dye as a result of its interaction with a specific polymeric structure. Thus, a large increase in anion site to dye ratios might cause no change at all or a change in either direction depending upon whether dye-dye interaction is a significant factor in the stoichiometric complex and whether the dye reaction caused some conformational changes in the polymer at low or high ratios.

The α, β, and γ and J-complexes could be readily accommodated under this hypothesis. The γ-complex appears, frequently, to be a transitory state, and it may be that this complex arises through direct adsorption of the dye aggregate, in the same form as it exists in the aqueous solution, onto the polymer. A subsequent rearrangement may then occur, in some cases, which results in a more stable relation of dye molecule to site.

Unfortunately, little can be said at the present time, about the intensely interesting S-state, since it has not been observed in a sufficient number of cases to permit fitting it into the complexing mechanism hypothesis. Since it appears occasionally as a secondary band under some conditions of excess site to dye relations, it is conceivable that it is another conformation related to the molecular J-band state.

The present studies have also reinforced some earlier observations that pH may have very little effect upon the formation of certain complexes. With amphoteric macromolecules, such as proteins, complex formation apparently may be prevented by a positively charged cationic group in the vicinity of the anion site. Changes in pH appear to have their effects mainly in altering such charges and permitting, or preventing the formation of the normal complex at the anion site. The polysaccharide complexes, involving carboxyl groups, are unaffected by alterations in pH which change the relative concentrations of their ionized and un-ionized groups by many orders of magnitude. To a lesser degree, this is also true of the polyphosphates. Thus, it appears that the degree of ionization of the participating anion group has little or no final effect upon the complex, either as a directive factor or in regard to the equilibrium between the complex and its component parts. It is possible, however, that an anion site may be required for initial reaction but, as the anionic sites are removed by the complexing, the ionized-non-ionized equilibrium is rapidly re-established among the remaining groups, leading to further reaction with the dye.

It may be concluded that it is unlikely that the complex metachromatic reactions of DBTC, or related systems, will be explicable in terms of simple mechanisms. Instead, it is probable that the complex states involve multiple interactions and conformational relations. Any interpretation must consider the interactions between the dye and binding site, between neighboring bound dye molecules and between neighboring binding sites as affected by the presence of the dye. It is particularly evident that the macromolecular binding site must be considered as a complicated unit in its interactions with the dye rather than a simple anionic site reacting with the cationic dye.

3.2 REACTION OF DYE WITH INORGANIC MOLECULES

It was established early in our research that inorganic materials could cause changes in the dye spectrum. Therefore, it was necessary to show that inorganic materials could be removed from the reaction mixture prior to addition of the dye. This was demonstrated in early work (Kay, 1963 and Bean, et al., 1964) by the removal of small molecular weight inorganic materials by ultrafiltration. As our attention focused more directly on the problem of detecting organic polymers in soils, it became clear that inorganic materials in general would have to be considered. A general approach was considered necessary because of the limited information available on the composition and environmental conditions on other planets. As an example, it would not be possible to exclude the presence of pyrophosphates on Mars, only because they are not detected in terrestrial soils and minerals. Work with silicates and polyphosphates revealed that simple ultrafiltration would not insure their removal prior to extraction with the dye.

The periodic table was surveyed for elements that can give rise to materials that could cause a change in the dye spectrum. As a result of this study a broad spectrum of chemicals was selected as representative of the various chemical types. Gimblett's text (1963) was employed as a guide in selection of inorganic polymers for test. The materials studied and tested were chosen because of their interference with the dye reaction in any of several ways, (a) formation of polymeric species in aqueous solution, (b) suspension as colloids, (c) difficulty in dialysis and (d) greater solubility in alkali and lower solubility at neutral pH. Aluminosilicates and silicates were one group selected on the basis of these criteria. Because of the ubiquity of aluminosilicates as components of the surface layers on earth it was of particular importance to study these minerals as possible interfering substances.

The purpose of this study of inorganic substances was to eliminate these compounds as sources of false positive information when testing for the presence of biological polymers with the dye system. This was to be accomplished by devising a procedure for their removal prior to introduction of the dye. It also became evident that much could be learned about the mechanism of the dye reaction by a study of the spectra obtained with inorganic polymers. In addition, if a proper sequence of operations were selected, the dye reaction could provide information in regard to a variety of both organic and inorganic substances. Some of the problems encountered and the procedures devised to overcome them in this study are discussed in this section of the report.

3.2.1 DYE REACTION WITH VARIOUS INORGANIC COMPOUNDS

The pure inorganic materials chosen for study were exposed to the standard soil extraction procedure as if they constituted the entire soil sample. The sample to solution ratio was 1:5 (w/v) in all cases. This procedure consists of (a) suspending the soil sample in acid and shaking, (b) separation of the soil from the acid, (c) suspending the soil in water or a chelating agent and shaking, (d) separation of the soil from the solution, (e) suspending the soil in alkali and shaking, (f) separation of soil from solution, (g) dialysis of the solution against alkali, and (h) finally, dialysis against water. This procedure is outlined in detail in Section 2 (Extraction of Inorganic Compounds), but it is important here to reiterate this sequence, as any of the inorganic compounds to be tested must be in the soil extract after the final water dialysis to cause a change in the dye spectrum. Some of the test compounds were completely soluble in acid at the concentration employed. However, many of the compounds required additional acid to bring the pH to 2.0. Sodium meta arsenite was completely soluble when suspended in 0.1N HCl, but the pH of the solution was 9.7. Upon adjustment to pH 2, a precipitate formed. The compounds soluble in acid included potassium pyrosulfite, potassium pyrosulfate, sodium arsenate, sodium pyrophosphate, sodium metaphosphate, sodium orthophosphate, phosphotungstic acid and

sodium hyposulfite. Since these materials were completely soluble in acid they could not interfere with the humic acid-dye reaction after the alkaline dialysis. Of the compounds not completely soluble in acid, only alum ($AlK(SO_4)_2$) was dissolved by the water wash. However, alkaline extracts of all the remaining compounds, potassium persulfate, sodium arsenite, ferric oxide, polysulfide, sodium metasilicate, potassium metasilicate, aluminum hydroxide and plumbous hydroxide gave no change in the dye spectrum after dialysis. Therefore, it can be concluded that the materials considered, if present in a soil sample, would not interfere with the detection of humic acid. As materials representative of a great number of inorganic compounds, they indicate that no interference should be encountered with these groups of compounds with the dye reaction after processing by the soil extraction procedure.

It was shown, however, that if some of these inorganic materials were not completely removed by previous steps in the extraction procedure, they could cause a change in the dye spectrum. The selected reagents as 20% mixtures (w/v) were shaken with water and the resultant supernate was tested with the dye to determine how it would change the dye spectrum. This approach was also followed with 0.5 N sodium hydroxide as extraction solution. However, the supernate of the alkaline extraction was dialyzed either against distilled water or against 0.01 N sodium hydroxide followed by distilled water. All samples were tested with dye for change in spectrum. Results obtained with the various compounds are shown in Table IV. All of the compounds prepared as solutions with distilled water caused a change in the spectrum of the dye and J-bands were obtained with $NaAsO_2$, $AlK(SO_4)_2$ and the $Na_2B_4O_7$. Dialysis against distilled water of alkali extracted compounds shows a significant decrease in the substances that gave a positive change in the dye spectrum. Of the samples which caused a change in the dye spectrum after water dialysis only the polysufide extract did not cause a change after both alkali and water dialysis. In the case of the potassium persulfate ($K_2S_2O_8$) extract, the situation was reversed. That portion of the extract subject to alkaline dialysis followed by water dialysis caused a change in the dye spectrum, while that portion subjected only to water dialysis did not cause a change in the dye spectrum. It is evident that alkaline extraction followed by water dialysis does not insure removal of inorganic compounds capable of causing a change in the dye spectrum.

Titration studies of $NaAsO_2$, $K_2S_2O_8$ and polysulfide with the dye were carried out to determine how sensitive the dye was to these compounds. Solutions of $NaAsO_2$ and $K_2S_2O_8$ were prepared 20% (w/v) in distilled water by shaking on a rotary shaker for 30 minutes. The $K_2S_2O_8$ and polysulfide suspensions were filtered to remove solid materials; this was not required for $NaAsO_2$ as it dissolved completely. Each of the solutions was serially diluted in distilled water. Samples of the various concentrations were combined with dye and the spectra recorded. Control spectra of the

TABLE IV

DYE REACTION WITH INORGANIC COMPOUNDS

Compound Tested	Pretreatment	Absorption Maxima Obtained: Compound in H_2O	Compound extracted with alkali & supernate dialyzed against: H_2O	Alkali + H_2O
$K_2S_2O_5$	None	540 mμ	dye peak*	-
$K_2S_2O_7$	None	610 mμ	dye peak*	-
$K_2S_2O_8$	None	545 mμ 590 mμ shoulder	dye peak*	510 mμ 560 mμ shoulder
$NaAsO_2$	None	630 mμ	540 mμ 670 mμ shoulder	510 mμ, 560 mμ shoulder
$AlK(SO_4)_2$	None	630 mμ	550 mμ 510 mμ	
$Na_4P_2O_7$	None	540 mμ	525 mμ 515 mμ	510 mμ
$Na_2S_2O_4$	None	540 mμ	520 mμ	510 mμ, 560 mμ shoulder
Fe_2O_3	None	520 mμ	525 mμ, 550 mμ shoulder	560 mμ 520 mμ
Polysulfide	None	-	520 mμ	dye peak*
H_3PO_4	None	630 mμ, 520 mμ	dye peak*	dye peak*
Phosphotungstic acid	None	540 mμ 590 mμ shoulder	530 mμ 590 mμ shoulder	510 mμ 590 mμ shoulder

* dye peak - spectrum was that given by the dye alone at the concentration employed indicating no change due to the inorganic material.

different samples with no dye added were also recorded. The results are presented in Table V. For solutions of all three compounds tested, considerable dilution was required before the dye spectrum was no longer affected. The dilutions required varied for the different compounds. Sodium meta-arsenite required less than a 100-fold dilution while the other two required higher dilutions. Therefore, these three compounds, which were not completely stable in the acid or the water wash steps of the soil extraction procedure, would apparently change the dye spectrum at relatively low concentrations. However, dialysis is effective in their removal.

3.2.2 REACTION OF DYE WITH MINERALS

Since the aluminosilicates are some of the most common minerals found on earth, and their colloidal suspensions can cause a change in the dye spectrum, it was essential to devise a procedure of soil extraction to eliminate interference from these substances. This was accomplished by a combined procedure of extraction and dialysis.

Some of the aluminosilicates selected for testing were montmorillonite (bentonite), Clay Spur, Wyoming; amphibole (uralite), Calumet, Colorado; phlogopite, Quebec, Canada [$K_4Mg_{12}Al_4Si_{12}O_{40}(OH)_8$], and kaolin. Since limonite had been suggested as a mineral possibly found on Mars by some researchers, two varieties of this mineral (limonite, Tuscaloosa County, Alabama, and limonite, var. yellow ocher - massive ($2\ Fe_2O_3 \cdot 3\ H_2O$) near Cartersville, Georgia) were also tested with the dye for possible interference in the macromolecule detection scheme.

Experiments were designed to determine whether the pretreatment and extraction solutions removed nondialyzable materials from minerals which cause a change in the dye spectrum. In designing these experiments there was no way in which dissolved and suspended aluminosilicate material could be differentiated. In either case, the material is effective in changing the spectrum of the dye.

The standard extraction procedure was followed with these minerals (see Section 2, Extraction of Minerals). This involved extraction of the minerals with various acid pretreating agents followed by extraction with alkali. Solutions were dialyzed either against alkali followed by distilled water dialysis or against distilled water only.

Table VI presents the results obtained when the dye was mixed with the pretreatment solutions and the water washes after they were dialyzed against distilled water. A significant shift in the dye spectrum is noted for all the dialyzed pretreatment solutions of the different minerals suggesting that some nondialyzable poly-anionic colloids were suspended or molecules were solubilized by the acid pretreatment step. The chemical nature of the silicates would tend to favor the supposition that the material was suspended rather than dissolved. Solution of some of the

TABLE V

DYE REACTION WITH SEVERAL INORGANIC COMPOUNDS

Compound Tested	Dilution	Absorption Maxima
$NaAsO_2$	10	650 mμ, 555 mμ
	10^2	dye peak, 570 mμ shoulder
	10^3	dye peak
$K_2S_2O_8$	10	dye bleached
	10^2	dye bleached
	10^4	dye peak, O.D. lower
	10^6	dye peak
Polysulfide	10^3	530 mμ
	10^4	dye peak

Solutions were prepared by extraction as 20% (w/v) in distilled water for 1/2 hour and were then filtered. The dye peak notation indicates that the spectrum of the dye alone was given.

TABLE VI

DYE REACTION WITH DIALYZED PRETREATMENT EXTRACTS OF MINERALS

Mineral Tested	Pretreatment or Wash	Absorption Maxima Obtained with Dialyzed Pretreatment Extract* Wave length in mμ
Kaolin	0.1M HCl	545
	H_2O wash after HCl pretreatment	520
	0.1M $Na_4P_2O_7$ pH 4.5	545
	H_2O wash after $Na_4P_2O_7$ pretreatment	530
	0.1M EDTA pH 4.5	545
	H_2O wash after EDTA pretreatment	510
Uralite	0.1M $Na_4P_2O_7$ pH 4.5	530
	H_2O wash after $Na_4P_2O_7$ pretreatment	510, 570 shoulder
Phlogopite	0.1M $Na_4P_2O_7$ pH 4.5	545
	H_2O wash after $Na_4P_2O_7$ pretreatment	545
Limonite (Georgia)	0.1M $Na_4P_2O_7$ pH 4.5	640, 525, 438
	H_2O wash after $Na_4P_2O_7$ pretreatment	640, 525
Limonite (Alabama)	0.1M $Na_4P_2O_7$ pH 4.5	512, increase at 570
	H_2O wash after $Na_4P_2O_7$ pretreatment	512, increase at 570

* The pretreatment extract was separated from the solid mineral and was dialyzed exhaustively against distilled water.

aluminum and other cations, however, may favor further suspension of the silicates. With iron oxides (limonites) the slightly acid pyrophosphate may be effective in solubilizing enough iron to permit colloids of iron to form during the dialysis against distilled water. All extracts of the aluminosilicate minerals caused the adsorption maximum to be shifted to the 530-540 mμ range. The yellow limonite with the acid extract gives a J-band (640 mμ), in contrast to the other limonite form tested (from Alabama), which caused the smallest shift in the dye spectrum giving essentially a dye peak with an increase in absorption at 570 mμ. Results with the water washes were somewhat varied, showing in most cases a smaller shift (than that observed with the acid solutions) of the dye spectrum from the free dye range. This indicates that the minerals tested were less soluble and/or less apt to be suspended as colloids by the water than by the acid pretreatment (HCl or pyrophosphate) step. The change in dye spectrum is not thought to result from incomplete dialysis of the pretreatment pyrophosphate. In the iron oxide extract the maximum absorption of the dye substantiates this and for the aluminosilicates the results were the same when hydrochloric acid was employed as the pretreatment solution.

Table VII shows the results obtained with the dialyzed NaOH extracts of the minerals tested (after pretreatment with several reagents). The most significant fact apparent from a study of the table is that all the minerals tested gave a free dye peak after acid pretreatment and dialysis against alkali and water. Figure 8 illustrates this result for uralite. After exhaustive water dialysis a J-band peak is still evident with the extract of this mineral. But, after dialysis against both alkali and distilled water, as can be seen in Figure 8, the dye-mineral extract spectrum approximates that of dye alone. Thus, acid pretreatment of the minerals followed by alkaline extraction and subsequent alkaline and water dialysis of the alkaline extract appears to be an effective procedure for eliminating the interference of inorganic substances. Inclusion of an isolation procedure including these steps in a soil organic macromolecule detection system introduces only one additional step. Acid pretreatment and alkaline extraction have been reported in the soil science literature (Choudhri and Stevenson, 1957) for maximum solubilization of soil organic matter. The only additional step in the proposed humic acid detection system is alkaline dialysis, which permits elimination of the inorganic materials soluble in dilute alkali and insoluble or converted to polymeric form at neutral pH. The necessity of this additional step is documented by the data in Table VII. When water dialysis alone is used, kaolin, bentonite and uralite all cause a change of the dye spectrum, apparently as a result of silicates. The particular pretreating agent used appears to be important. Phlogopite, pretreated with 0.1 M $Na_4P_2O_7$ at pH 4.5 yielded an alkaline extract which, after alkaline and water dialysis, caused a change in the dye spectrum with a major peak observed at 540 mμ. But when 0.1 N HCl was used prior to alkaline extraction, no new peak was observed with the alkali or water dialyzed extracts (Fig. 9). However,

TABLE VII

DYE REACTION WITH DIALYZED SODIUM HYDROXIDE EXTRACTS OF MINERALS

Mineral Tested	Pretreatment Used	Absorption Maxima	
		H_2O Dialysis	Alkaline & H_2O Dialysis
Kaolin	0.1M HCl	540 mμ	dye peak
	0.1M $Na_4P_2O_7$ (pH 4.5)	540 mμ	510 mμ, increase at 570 mμ
	0.1M EDTA (pH 4.5)	540 mμ	510 mμ, increase at 570 mμ
	None	630, 550, 485 mμ	540 mμ
Bentonite	0.1M HCl	dye peak	dye peak
	0.1M $Na_4P_2O_7$ pH 4.5	625, 480 mμ	dye peak
	None	dye peak	dye peak
Uralite	0.1M HCl	dye peak	dye peak
	0.1M $Na_4P_2O_7$ pH 4.5	630, 480 mμ	dye peak
Limonite (Georgia)	0.1M $Na_4P_2O_7$ pH 4.5	dye peak	dye peak
Limonite (Alabama)	0.1M $Na_4P_2O_7$ pH 4.5	dye peak	dye peak
Phlogopite	0.1M HCl (.22 μ filter)	dye peak	dye peak
	0.1M HCl (.1 μ filter)	530 mμ	dye peak
	0.1M $Na_4P_2O_7$ pH 4.5	540 mμ	540 mμ
Sodium Silicate (Na_2O/SiO_2, 1/2.86)	0.1M HCl	500 mμ	dye peak, 600 mμ shoulder
	None	495 mμ	dye peak
Potassium Silicate (K_2O/SiO_2, 1/2.1)	0.1M HCl	500 mμ	dye peak, 580 mμ shoulder
	None	495 mμ	dye peak

The dye peak notation is used to indicate that the spectrum of the dye alone was given, except where change is indicated as a shoulder.

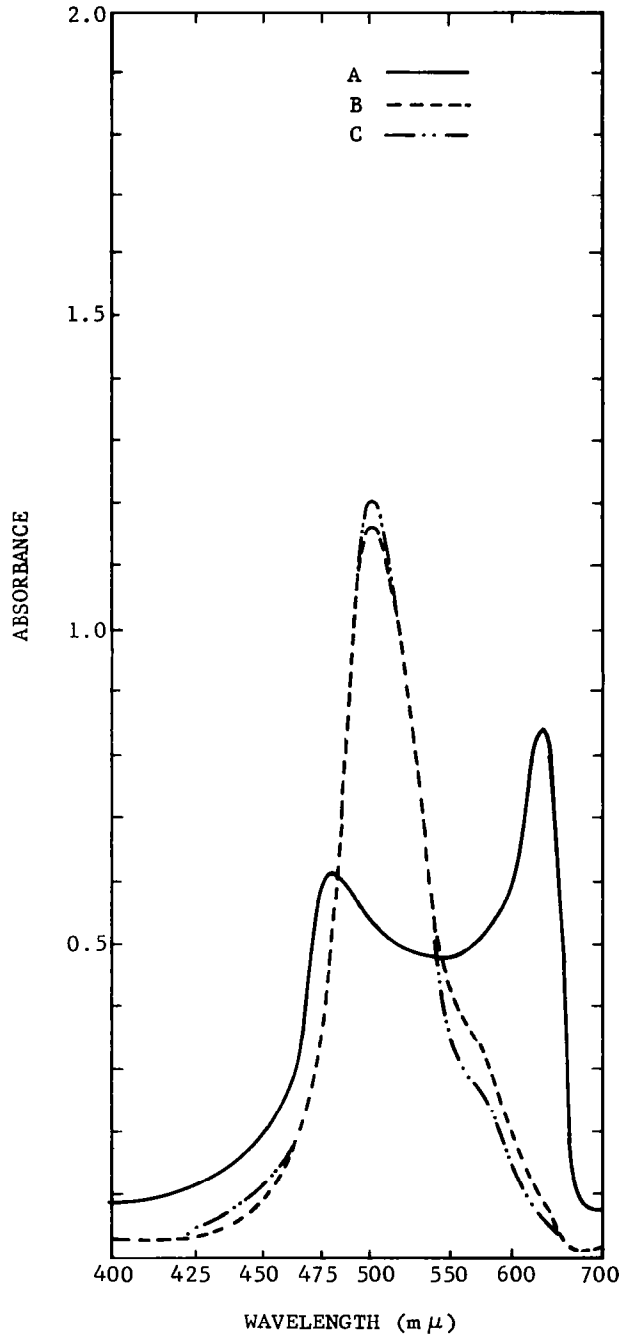

FIGURE 8. REACTION OF A DIALYZED EXTRACT OF URALITE WITH THE DYE.

The uralite was extracted as a 20% suspension (w/v) in 0.5 N sodium hydroxide after pretreatment with 0.1M $Na_4P_2O_7$ at pH 4.5. After centrifugation the extract was divided into two portions, one part being dialyzed exhaustively against distilled water while the remainder was dialyzed against 0.1N alkali and then against distilled water. The samples were then mixed with dye to give a final dye concentration of 2×10^{-5}M. A is the spectrum obtained with the water dialyzed sample while B is the spectrum obtained with the alkali and water dialyzed sample. Curve C, the free dye spectrum, is shown for comparison.

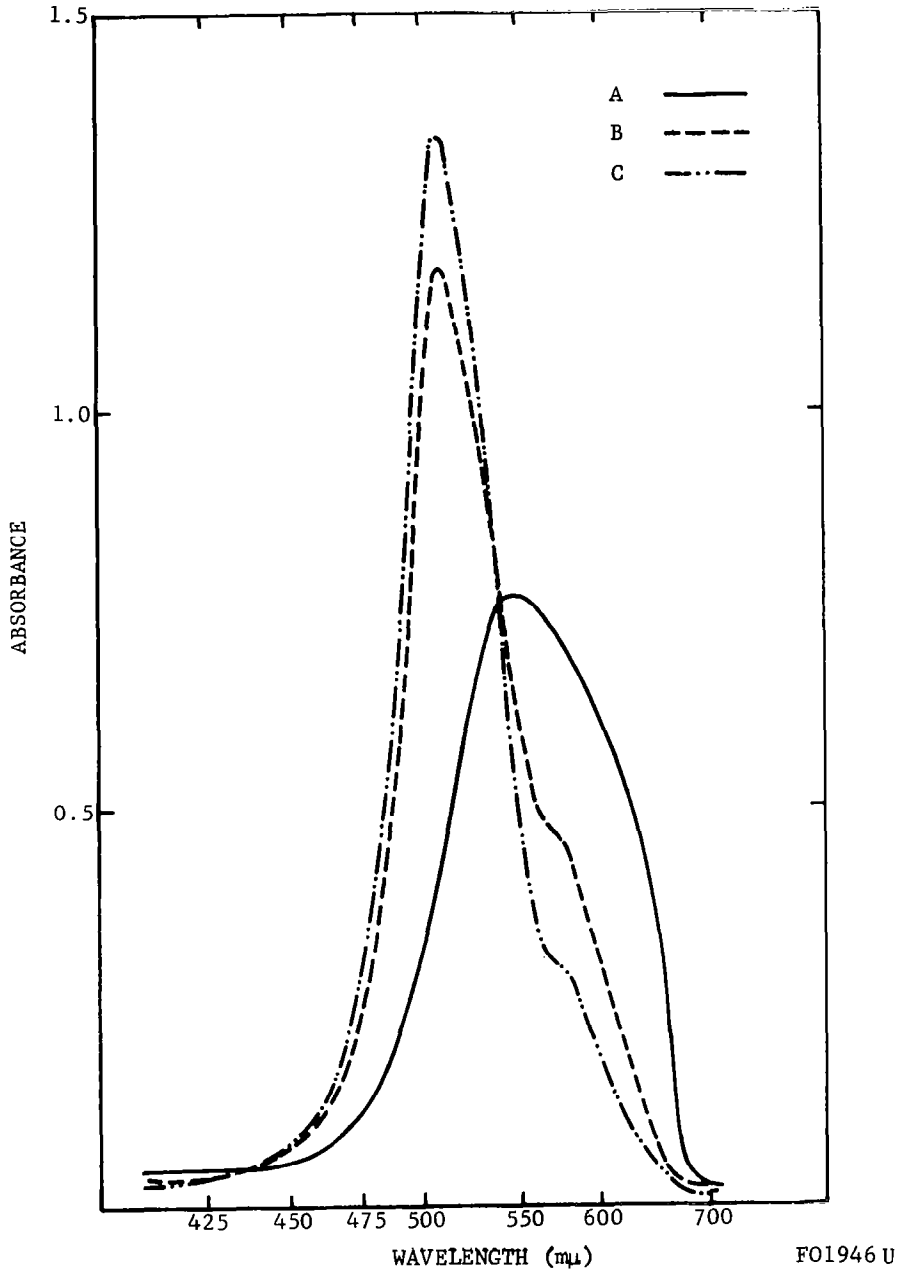

FIGURE 9. REACTION OF A DIALYZED PHLOGOPITE EXTRACT WITH THE DYE.

The phlogopite was extracted as a 20% suspension (w/v) in 0.5N sodium hydroxide after pretreatment with either 0.1M $Na_4P_2O_7$ at pH 4.5 or 0.1M HCl. After centrifugation the extracts were dialyzed against 0.1N alkali and then against distilled water. Dye concentration was 2 x 10^{-5}M in the final mixture. Curve A is the sample pretreated with the $Na_4P_2O_7$, B is the spectrum obtained with the 0.1M HCl treated sample while Curve C is the free dye spectrum.

this effect may also be due to a variation in the procedure used in the experiment where 0.1 M HCl was used to pretreat. In this case, the phlogopite was first broken into thin plates then converted into a light powder by grinding in a Waring blendor. In the earlier experiment where 0.1 M $Na_4P_2O_7$ was used for pretreatment, the mineral was extracted as thin small sheets (previously ground in a mortar) providing a lower surface to mass ratio for the pretreating reagent.

Results presented in Table VII show that the extracts of limonite (suggested as common to the surface of Mars) caused no change of the dye spectrum for either the water or alkali and water dialyzed extracts.

The period of alkaline dialysis in the treatment of the various minerals seemed to be critical for some of the minerals tested. A dialysis study was carried out to determine the minimum time required for alkali dialysis to remove silicate materials. The dialysis was carried out as a batch process using beakers and magnetic stirrers in the dialysis procedure with a set number of changes in the dialysis solution. It is reasonable to assume that the dialysis time required could be further decreased by application of a dialysis system permitting continuous removal of the silicate from the dialysate.

In this experiment the various minerals were pretreated with 0.1 N HCl and washed with distilled water. The extraction was completed with 0.5 N sodium hydroxide.

After centrifugation the solutions were filtered through 0.22 μ Millipore filters, however, for one phlogopite solution a 0.1 μ pore filter was used. Each extract was dialyzed against 0.1 N NaOH for 1, 2, 3 or 5 days using 4000 ml beakers with a change of alkali every 24 hours. The extracts (retentates) were then exhaustively dialyzed against distilled water. Controls consisted of 0.5 N NaOH treated in the same manner as the mineral extracts. All the dialyzed solutions were then combined with the dye and the spectra recorded. For comparison, experiments were also carried out following the same procedure except no pretreating agent was used. The results of this work are shown in Table VIII.

A study of this table shows that free dye spectra were obtained with all of the minerals tested after pretreatment and alkaline dialysis for two days. When no pretreatment was used with kaolin before alkaline extraction of this mineral, a J-band was observed with the dye if only distilled water dialysis was employed. Alkaline dialysis caused loss of the J-band peak, but even after five days of dialysis the dye spectrum with the extract was not that of the dye alone (Fig. 10). However, when the kaolin was extracted with an acid pretreatment a free dye peak was obtained after 2 days of alkaline dialysis (Fig. 11).

TABLE VIII

TIMED DIALYSIS STUDY OF MINERALS

Sample	Time of alkaline dialysis in days	Absorption Maxima Wave length in mμ	O.D.
Control*	0	508	1.32
	1	509	1.37
	2	508	1.34
	3	508	1.38
	5	508	1.32
Control*	0	505	1.21
	1	504	1.35
	2	505	1.12
	5	505	1.23
Kaolin	0	545, 650 shoulder	0.54
(0.1N HCl pretreatment)	1	530	0.94
	2	512	1.06
	3	510	1.11
	5	510	1.11
Kaolin	0	650, 540, 488	0.88, 0.57, 0.56
(No pretreatment)	1	545	0.85
	3	540	0.89
	5	528	0.92
Bentonite	0	508	1.32
(0.1N HCl pretreatment)	1	508	1.26
	2	505	1.32
	3	510	1.29
	5	506	1.35
Bentonite	0	505	1.14
(no pretreatment)	1	508	1.08
	3	505	1.26
	5	505	1.17
Phlogopite	0	510	1.13
(0.1N HCl pretreat-	1	511	0.96
ment 0.22 μ filter)	2	512	1.03
	3	510	1.12
	5	510	1.18

TABLE VIII, Cont'd

Phlogopite	0	530	0.95
(0.1N HCl pretreat-	1	520	0.97
ment 0.1 μ filter)	2	511	1.04
	3	510	0.98
	5	510	1.02
Uralite	0	505	1.34
(0.1N HCl pretreatment)	1	505	1.42
	2	505	1.41
	3	507	1.36
	5	505	1.33
Sodium silicate	0	495	1.33
(Na_2O/SiO_2, 1/2.86)	1	505	1.34
(no pretreatment)	2	505	1.35
Potassium silicate	0	493	1.30
(K_2O/SiO_2, 1/2.1)	1	502	1.50
(no pretreatment)	2	505	1.28
	3	505	1.33

* Control consisted of an aliquot of the 0.5N NaOH used in extraction of minerals. The sodium hydroxide solution was then processed as the supernates from the mineral extractions.

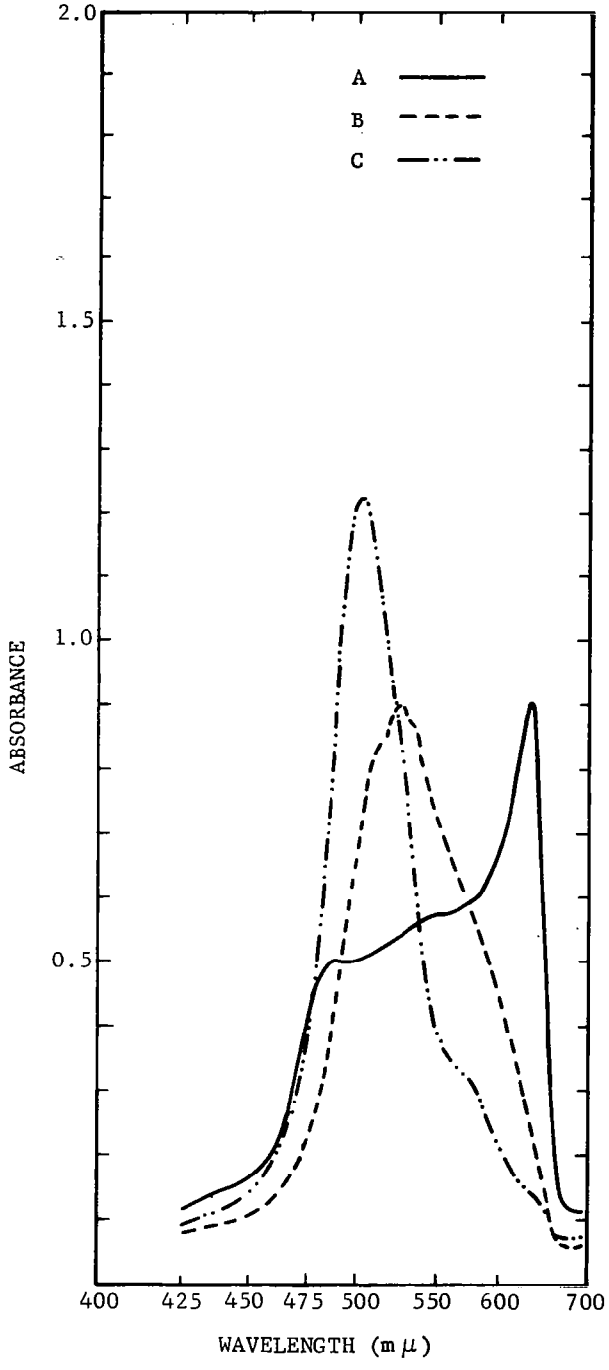

FIGURE 10. REACTION OF A DIALYZED KAOLIN EXTRACT WITH THE DYE.

The kaolin was extracted as a 20% suspension (w/v) in 0.5N sodium hydroxide. After centrifugation the extracts were divided into two portions, one part being dialyzed exhaustively against distilled water, while the remainder was dialyzed against 0.1N alkali and then against distilled water. The samples were mixed with dye to give a final dye concentration of 2×10^{-5}M. A is the spectrum obtained with the water dialyzed sample, B is the spectrum for the water and alkali dialyzed sample, while C is the free dye spectrum shown for comparison.

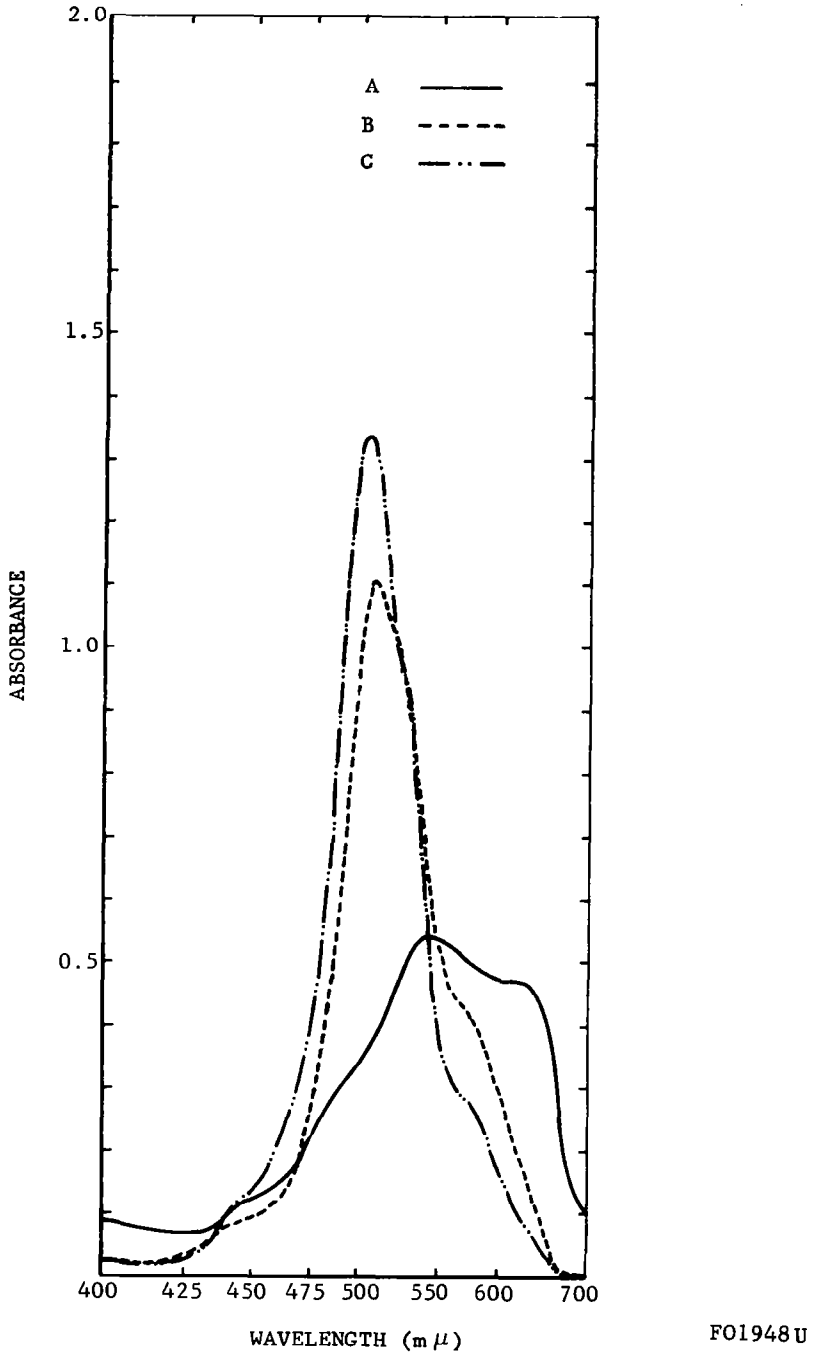

FIGURE 11. REACTION OF A PRETREATED KAOLIN EXTRACT WITH THE DYE.

The kaolin was extracted as a 20% suspension (w/v) in 0.5N sodium hydroxide after pretreatment with 0.1M HCl. After centrifugation the extracts were divided into two portions, one part being dialyzed exhaustively against distilled water, while the remainder was dialyzed first against 0.1N alkali and then against distilled water. The samples were mixed with dye to give a final dye concentration of 2×10^{-5}M. A is the spectrum of the water dialyzed sample, B is the curve of the alkali and water dialyzed sample and C is the free dye spectrum.

3.3 HUMIC ACID

Humic acid is a substance characterized by definition rather than by exact chemical composition. This situation results from the complexity of this material and the variety of its constituent components. In this study we have defined humic acid as that organic material which can be extracted from the soil, is soluble in alkali and does not dialyze. This definition does not deviate substantially from the classic definition of humic acid which is that material soluble in alkali, but precipitated by acid. The component of humic acid in which we have been most interested, with respect to its reaction with the thiacarbocyanine dye, is the material characterized as polyphenolic, conjugated, unsaturated and containing hydroxyl, carboxyl and carbonyl groups. This material constitutes a majority of the humic acid and is to be contrasted with the minor quantities of nucleic acid, protein and carbohydrate which are present as polynucleotides, peptides or polysaccharides.

In early chemical characterization of humic acid considerable effort was expended in elucidating the basic monomeric unit of humic acid (Kononova, 1961, pp. 65-66). This work did not prove too successful because of variations with different samples. Nevertheless, the matrix of humic acid appears to be consistently composed of condensed phenolic groups with the functional groups mentioned previously, and this appears to be common to all soil humic acids.

3.3.1 EXTRACTION

The percent of the organic material which can be extracted from a soil varies with the extraction procedure and the solutions used for extraction. The percent of soil organic material which can be extracted,also, varies with the type of soil extracted. In our early work a single step extraction was investigated for its utility in extraction of soil organic material. Results from this work and the work of others were interpreted as indicating that a single extraction step did not give a broad enough potential to deal with the great variety of soil types which can be encountered. For this reason extraction sequences were investigated. These sequences involved pretreatment of the soil sample with a chelating agent to remove di- and trivalent cations which, it is suspected, act to bind the organic material to the inorganic materials of the soil particle. However, it was desirable that this chelating agent not extract any appreciable amount of the humic acid. Therefore, this pretreatment was carried out at a slightly acid pH of 4.5 and should only solubilize fulvic acid. The results of such a study with a local agriculture soil are shown in Table IX. Briefly the extraction procedure consisted of suspending the soil in the pretreatment solution for 1 hour with shaking. The soil was separated by centrifugation, washed five times with water and shaken in 0.5 N NaOH for one hour. The soil residue was separated from the extract by centrifugation. The supernate

TABLE IX

EXTRACTION OF ORGANIC CARBON FROM A CLAY SOIL

Pre-treating Agent	Percent of Total Soil Organic Carbon Removed by pre-treatment (+ washes)	Removed by 0.5N NaOH (+ washes)	Removed by NaOH and non-dialyzable
None	---	18	3.0
H_2O	None	34	1.4
.1M HCl	21	15	3.2
.25 M lactic acid (pH 4.3)	15	40	10.4
.1M EDTA* (pH 4.5)	9	20	4.9
.1M pyrophosphate	None	54	4.8
.3M HCl-HF followed by 0.1M pyrophosphate (pH 4.5)		21	7.4

Soil employed in these studies was a local agricultural clay containing soil. The organic carbon content of this soil is 0.85 percent.

* Ethylenediaminetetra acetic acid

was exhaustively dialyzed against distilled water and the precipitate (extracted soil) was dried and analyzed for carbon. Several interesting points are apparent. The pyrophosphate pretreatment removed no carbon. This is an ideal result for a pretreatment, since it results in no loss of organic material. Because of this favorable result, the experiment was repeated and the previous result confirmed. It is important to note the variation in the amount of organic carbon extracted by the different extraction solutions, particularly the differences in the percent of dialyzable material. Examples of this are seen in the results with EDTA and pyrophosphate. Pyrophosphate removed 54% of the total organic carbon but only 4.8 was nondialyzable, with EDTA only 20 (or a total of 29) percent was removed but 4.9 percent was nondialyzable. The organic carbon removed by the lactate pretreatment plus the subsequent sodium hydroxide extraction was roughly the same as that removed by the sodium hydroxide extraction of the pyrophosphate pretreated soil, but there was twice as much nondialyzable carbon removed by the lactate system.

The results show that with the exception of water, each of the pretreatment schemes was effective in increasing the amount of nondialyzable organic material extracted by 0.5N NaOH. The results with pyrophosphate demonstrate that a pretreatment can be used which will not remove organic material from the soil, but will aid in increasing the amount of material subsequently extracted with alkali. It was anticipated that this would be the type of result obtained with the HCl pretreatment, on the basis of the results obtained by Choudhri and Stevenson (1957). Their results for four clay type soils indicated HCl pretreatment greatly increased (3 fold in one case) the humic acid solubilized, but did not greatly increase the total carbon extracted. Our results contrasted with those of Choudhri and Stevenson (1957) point up the problems of attempting to devise a universal soil extraction procedure. The main point to be made is the appropriateness of a pretreatment prior to alkali extraction. It was felt that clay containing soils offer the greatest difficulties to the extraction of organic materials and also interference with the dye reaction. It was believed that work with the clay soils could serve as a reference for work with desert soils.

The last entry shown in Table IX involved the use of two pretreatment steps. These included 0.3 M HCl-0.3M HF and pyrophosphate. The HF-HCl mixture was employed to permit solution of silicates. The exposure to this solution is for only 10 min. at 4°C, therefore extensive dissolution of the silicate is not obtained, but rather solution of the readily soluble silicate, particularly that associated with the humic acid. Roughly one and one-half times as much extractable, nondialyzable carbon is obtained by adding the HF-HCl treatment. The result for the dialyzed sample can not be compared directly with the other values as it was dialyzed first against alkali and then distilled water. This treatment permits dialysis of extracted materials during the alkali dialysis that were precipitated by the rapid decrease of pH which occured with the other samples during dialysis against distilled water.

In later soil extraction studies the HCl-HF pretreatment combined with a subsequent pyrophosphate pretreatment was utilized. The reason for this, in spite of better extraction of organic carbon with lactic acid, was the elimination of an introduction of extraneous carbon compounds, which might affect the organic carbon analysis performed on the various fractions and the final dialyzed product. The scheme followed for this isolation is shown in Figure 12. Concentration and additional alkaline dialysis was considered necessary to obtain sufficient sample for the work and to obtain adequate dialysis of the final sample. When this scheme was employed with the Mohave desert soil 76-2, (organic carbon 0.4 percent*) the sodium hydroxide solution contained 12 percent of the soil organic carbon. Dialysis against alkali and water gave a nondialyzable fraction, which contained 8.5 percent of the soil organic carbon. The organic carbon to solids ratio for the dialyzed sodium hydroxide sample was 1:2.0. This extraction procedure with the agricultural clay soil (Mesa Verde) gave an organic carbon to solids ratio of 1:61 for the neutralized sodium hydroxide extract (after correction for sodium chloride) and after the dialysis treatment it gave a ratio of 1:2.6. The yield of the organic carbon with the 76-2 soil was surprisingly low. However, it is important to remember that the conditions of the extraction were designed to limit the degradation of the organic materials. In addition, the 76-2 soil contains a considerable amount of clay material, which makes extraction more difficult. The results from this work again indicate the difficulties in designing a universal soil extraction procedure.

During this work considerable effort has gone into demonstrating that the dye was reacting with humic acid and not inorganic material associated with it. The major contaminant of concern in this respect is silicate dissolved during alkaline extraction. One approach used to demonstrate this point involved wet oxidation of the carbonaceous matter by the Allison procedure (acid digestion with 57% sulfuric and 34% phosphoric acids, before the addition of dichromate) used in carbon analysis of soils and extracts. The acid was neutralized after oxidation of the extract and small molecular weight compounds removed by dialysis. However, during neutralization at least a 1:200 dilution was necessary to prevent visible precipitation. Exhaustive dialysis resulted in a cloudy, yellow suspension and a dark-colored precipitate. It was, therefore, concluded that this method of oxidation presented too many experimental problems to warrant further consideration. Furthermore, there was no assurance that the concentrations and structures of inorganic macromolecules present would remain unchanged during this oxidation procedure.

In early studies another approach was used in which organic material was removed from soil prior to alkaline extraction by incineration of the sample. Although this procedure eliminated organic materials, the disadvantage of this approach (as evidence for lack of interaction of the dye with inorganic polymers in soil extracts) is that heat can change the

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* The organic carbon for this soil was earlier reported as 0.25 percent. An error was discovered which puts the corrected value at 0.4 percent.

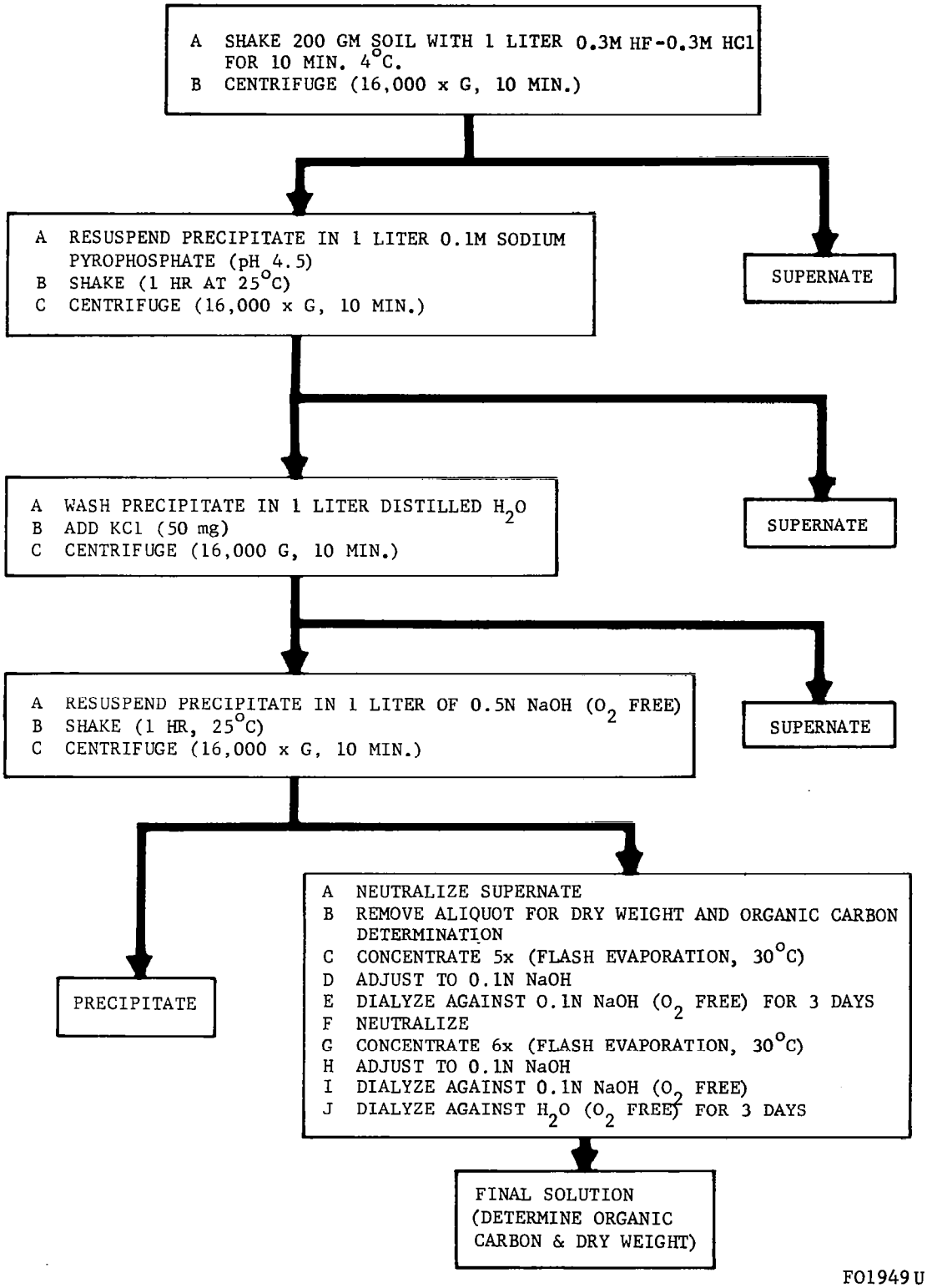

FIGURE 12. SOIL EXTRACTION FLOW CHART

nature of the inorganic constitutents of the soil and the rate of solution of the silicates. The earlier work with incinerated soils showed that extracts of incinerated soils did not cause significant changes in the dye spectrum. In this incineration procedure the soil temperature was about 700°C. The sample was heated in a crucible with a laboratory burner. This temperature is sufficient to soften Corning soft glasses and it is well above their annealing temperature of 550°C. Although silicate formation by sintering of orthosilicate is usually carried out between 700 to 1500°C, some natural calcium silicates are formed in aqueous solution even at temperatures of less than 400°C (Hersh, 1961). However, the physical properties of alkali metal silicates and commercial aluminosilicates (Hersh, 1961, Weinheim, 1959, Vail, 1952) strongly suggests that similar constituents found in soils are free of melting, fusing and glass-forming elements (but not crystal structure changes) up to at least 570°C. Therefore, it appeared that controlled heating at temperatures of 550°C would permit oxidation of the organic matter without affecting the silicate material. This approach was further supported by recent work (Schnitzer and Hoffman, 1964) which showed that soil samples heated to 540°C lost all oxygen from the functional groups of the organic matter. Therefore, in samples heated to this temperature, the hydrocarbon char or residue would be expected to have no oxygen containing functional groups and would not interact with the dye, even if it were extracted by alkali.

Soil samples were heated for 10 and 60 minutes in covered procelain crucibles in a muffle furnace at 500-520°C. After cooling, the samples were extracted with 0.5 N NaOH, followed by exhaustive dialysis of the extracts before examining their ability to change the dye spectrum. This heating procedure was also carried out on the nondialyzable portions of dried soil extracts. After heating, the residue was extracted with hot water to ensure extraction of any silicate which may be present.

A soil sample (Tierra Loam 1.75% organic C) was spread out on the sides of a porcelain crucible, covered, and heated to 520°C in a muffle furnace. After 10 minutes at 500-520°C the black soil was cooled to room temperature and extracted with 0.5 N NaOH and centrifuged. The supernate was a dark reddish-brown solution, indicating a relatively high concentration of organic matter, just as in an extract of unheated soil. In contrast, a soil sample maintained at the 500-520°C pyrolysis temperature range for one hour, was brown in color. The supernate of the alkali extract of this 1-hr.-pyrolyzed sample was a clear, very light yellow solution. Carbon analysis of this solution indicated that 7.5% of the organic C (present before pyrolysis) was extracted. These extracts were dialyzed against a solution of 0.01 N NaOH which retained a pH above 12 during the one day dialysis. This pH was maintained to ensure the dialysis of dissolved silicates. The extract from the 1 hr. pyrolysis after one day further dialysis against distilled water was colorless while that from the 10 min. pyrolysis had color. These solutions were individually combined with the dye and their spectra determined. The results are shown in Figure 13. They show the similarity between the

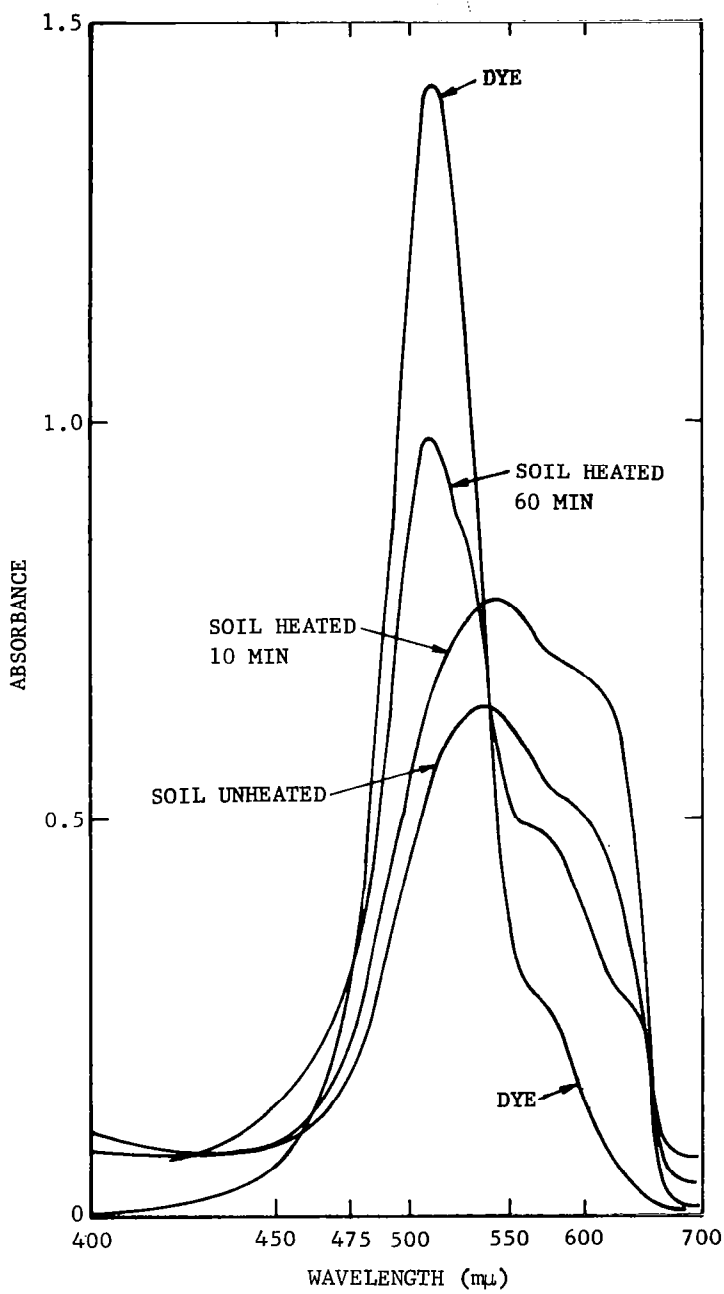

FIGURE 13. DYE REACTION WITH EXTRACT FROM SOIL SUBJECTED TO CONTROLLED TEMPERATURE PYROLYSIS.

Soil W (Tierra Loam) was subjected to pyrolysis at 520°C for the time indicated. It was then extracted with alkali and dialyzed against alkali and water.

10 min. heated sample and the unheated sample. It is evident that even after 1 hr. pyrolysis at this temperature that extractable material remains which reacts with the dye. The controlled temperature pyrolysis was effective in removing functional groups from the soil organic matter. It demonstrated that at pyrolysis temperatures which should not affect the silicates there is a definite decrease in the amount of dye reactive material and therefore the dye reactive material must be largely organic in nature. It is also apparent that the reaction does not quantitatively remove the functional groups from the organic material. This contrasted to some extent with the results obtained by pyrolysis of the nondialyzable soil extract, in which no dye reactive material could be extracted after pyrolysis. This pyrolysis of the nondialyzable portions of dried soil extracts was carried out in a muffle furnace heated to 520°C. A dried extract of Tierra Loam was heated just to this temperature and cooled. Extraction of the black char which resulted was made with hot water. Reaction of the water extract with the dye caused a change in the spectrum which was qualitatively about the same before and after this heating. When the pyrolysis temperature was maintained for about 10 minutes, the heated soil extract material was a brown, filamentous char. After hot water extraction, filtration and dialysis, the colorless solution did not interact with the dye, as indicated by the spectrum which was like a diluted dye spectrum. However, in this case it is likely that the carbonized material would prevent effective extraction of partially decarboxylated humic acid.

The last and most successful approach to this problem of determining whether soil silicates are interfering with detection of soil organic material involves the direct assay of silicate with a color determination of a silico-molybdate complex. The purpose of this was to show that the silicates extracted by the alkaline extraction could be removed by a subsequent alkaline dialysis.

In an initial experiment an extract of soil 76-2, a Mojave Desert soil, was prepared by the standard soil extraction procedure as described in Section 2. Pyrophosphate (pH 4.5) was employed for pretreatment. Twenty-five ml of this extract was taken for silicate analysis and the remainder of the extract was concentrated 3.5 times by flash evaporation. This extract was dialyzed against 0.1 N sodium hydroxide for two days, followed by exhaustive water dialysis. The dialyzed and undialyzed soil extracts (as well as a standard for use as a control) were treated with a 30% solution of hydrogen peroxide and heated until the solutions were clear. This step was carried out in order to obtain a clear solution that would not interfere with the silicomolybdate color reaction, since the soil extract itself had a dark brown color. After cooling of the solutions, catalase was added until foaming was no longer evident in the solutions showing that reduction of the peroxide had taken place. The extracts (both the dialyzed and undialyzed) were analyzed for the presence of silicate by use of a colorimetric test based on the formation of a silicomolybdate complex, as described in Section 2.

The results obtained from the silicate test are evident in Figure 14. The standard silicates (both the peroxide treated and the untreated) both show about the same optical density, verifying that the peroxide treatment did not interfere with the colorimetric silicate test. The undialyzed soil extract showed a significantly higher optical density comparable to that of the 0.025 mg silicate standard. But the same soil extract, after being subjected to alkaline and water dialysis had approximately the same optical density as the blank. This result indicated that alkaline dialysis is effective in removing silicates from soil extracts.

Subsequent work involved quantitative determination of silicate in the final soil extract preparations. Extraction of the agricultural clay (Mesa Verde) and the desert soil 76-2 was carried out according to the procedure outlined in Figure 12. The final dialyzed products of this work were more concentrated than extracts obtained by the standard extraction procedure of 2 gm of soil mixed with 10 ml of extraction solution. This concentration would present more of a problem in eliminating the extracted silicate by alkaline dialysis. The level of concentration was 16 fold for the Mesa Verde soil and 30 fold for the 76-2. Silicate standards were made up by fusion of sodium carbonate and silicic acid and a standard curve constructed. The soil extracts were heated with hydrogen peroxide to decolorize the solution and the excess hydrogen peroxide degraded with catalase. Standards were also subjected to the heating-hydrogen peroxide and catalase treatment. The concentration of silicate in both soil samples was the same 0.09 mM. We previously reported (Bean, et al., 1964) that the dye spectrum was not affected by silicate concentrations at less than 0.2 mM Si. However, it is important to remember that these soil extracts were concentrated. In the following section (3.3.2) results of titration of these soil extracts with the dye will be presented. These results show (Figure 15 and 16) that over a 1500 fold dilution of either dialyzed soil extract could be made and still obtain a complete titration of the dye (i.e., the spectrum of the dye altered from a peak at 510 mμ to one at 535 mμ.) This dilution would reduce the silicate concentration to roughly 0.06 μM. The change of dye spectrum which is seen with the soil extract at this dilution could hardly be due to traces of silicate at this concentration. Therefore, there can be little question that the dye reacts with the soil organic material and not a trace of inorganic material.

3.3.2 CHEMISTRY

The basic purpose of the research reported in this section was to determine with what functional groups of the humic acid the dye reacts. The extracts used in this work were from two soils, 76-2 and Mesa Verde. The processes which were investigated in this respect involved various physical measurements and chemical modification. Data on the reaction of the dye with the soil extracts is first presented and the results related to the findings in the dye reaction mechanism studies.

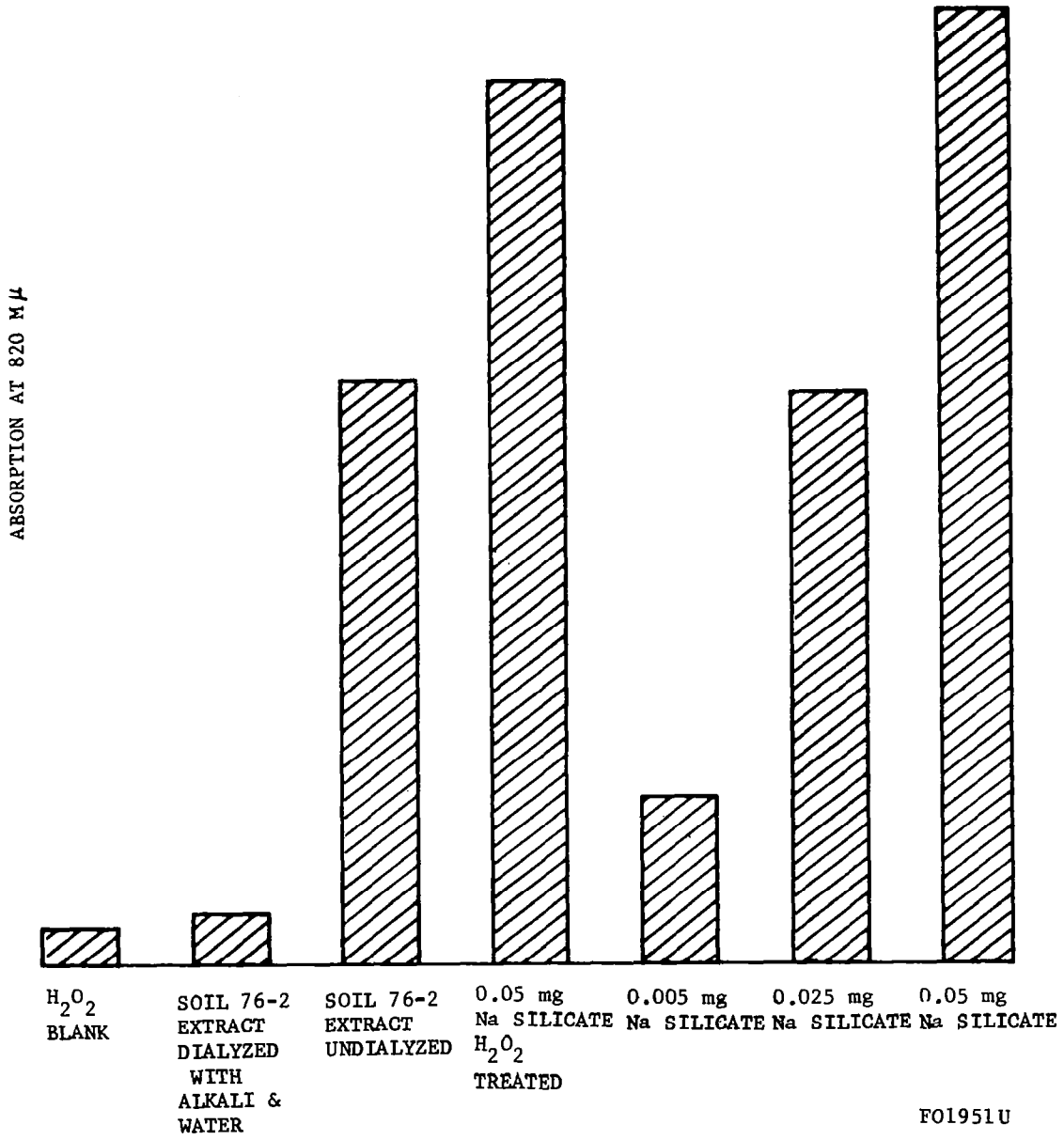

FIGURE 14. SILICATE CONTENT OF A SOIL EXTRACT

A sodium hydroxide extract of soil 76-2 was analyzed before and after dialysis against alkali and water for silicate by use of a colorimetric test (Furman, 1962). The absorptions at 820 mμ are compared for the standards and the samples.

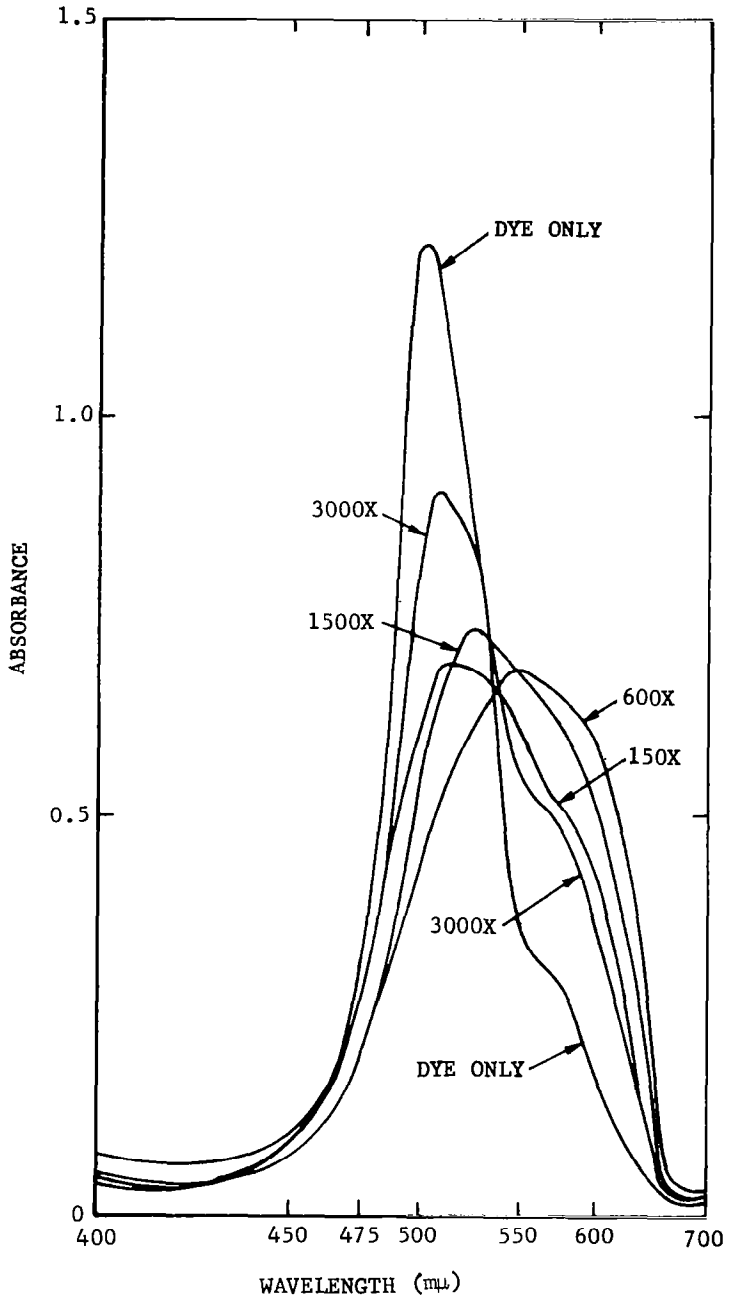

FIGURE 15. TITRATION OF DESERT SOIL EXTRACT WITH DYE.

The soil (Mohave Desert Soil 76-2) was extracted according to the outline in Figure 12. The dialyzed extract was diluted and combined with the dye and buffer solutions to give the final dilutions shown. Final concentration of the dye was 2×10^{-5} M in all cases.

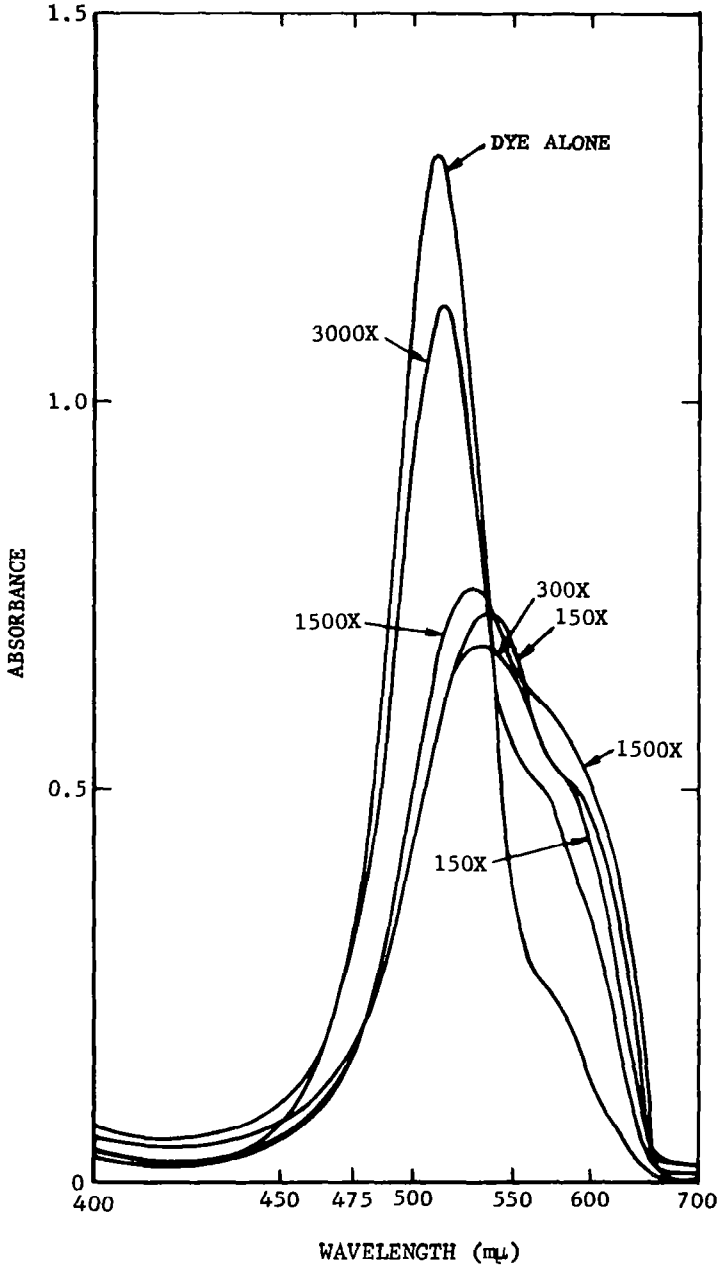

FIGURE 16. TITRATION OF SOIL EXTRACT WITH DYE.

The Mesa Verde soil was extracted according to the procedure in Figure 15. The dialyzed extract was diluted and combined with the dye and buffer solutions to give the final dilutions shown. Final concentration of the dye was 2×10^{-5} M in all cases.

Results of the dye titration of the soil extract preparations for the two soils are shown in Figures 15 and 16. For both extracts it can be seen that a dilution of 1500 times can be made before there is much of an increase at 510 mμ. An interesting effect seen with the 76-2 soil extract is the shift in maximum from 510 mμ at high concentration (150 x dilution) to 535 with a dilution of 600 x. In making calculations for the dye titration the procedure outlined in Section 3.1 was used. However, since there is a decrease and then an increase in the absorbance at 510 mμ, the minimum value is considered the equivalence point. For the four samples (two for each soil) to be compared in the ensuing work the dye reactivity is related to the organic carbon of the individual samples. This was accomplished by determining the minimum dilution of the dialyzed soil extract required to obtain a minimum absorption at 510 mμ, (the peak of absorption of the unreacted dye). The organic carbon concentration at this dilution is computed and divided by the dye concentration (2×10^{-5} M) to give gm of organic carbon per dye equivalent. The results from such a determination are given in Table IX. It was rather surprising that the average equivalent weights of the extracts from the two soils were about the same. The values obtained also indicate a relatively small unit weight per dye reactive functional group, on the order of 12 carbons per functional group or two phenyl radicals. The assumption is made that the humic acid functional group to dye molecule ratio is 1:1 or larger as indicated by our results with other polymers such as polygalacturonic acid, heparin, polyaspartic acid, polyglutamic acid. However, all these polymers contain carboxyl groups as functional groups. It is well established that humic acid contains phenolic groups. At present we do not have data on the reactivity of the dye with acid phenolic groups of polymers, but the preceding results suggest some reactivity of the dye with these groups. The titration results, also, demonstrate the type of spectrum obtained when humic acid is combined with the dye. This type of complex was designated the β type in Section 3.1. Other polymers of this group include polyaspartic, polyadenylic, denatured DNA, heparin and metaphosphate. By comparison this would suggest that the anionic groups on humic acid are closely and regularly spaced. Earlier work reported (Bean et al., 1964) that material which could give a J type spectrum could be obtained from humic acid after chromatographic fractionation, but the results would suggest this was a minor component of the humic acid. All unfractionated humic acid preparations so far studied gave the β and βa complex type spectrum.

The results of the dye titration of the soil extract also lend themselves to a calculation of the sensitivity of the detection system. In the case of the sample from soil 76-2 (Figure 15) at least a two-thousand-fold dilution of the sample could be made. The concentration of the organic carbon in this sample is 1.9 mg/ml (Table X, 76-2 sample 1) before dilution. This would mean 1.0 μg is detected with the routine laboratory procedure. However, this is not the ultimate sensitivity as the dye concentration can be decreased several fold to give increased sensitivity and useful spectrum changes. In addition, the light path of the spectrophotometer cuvette can

TABLE X

DETERMINATION OF DYE EQUIVALENCE FOR DIALYZED SOIL EXTRACTS

Soil	Dialyzed Soil Extract: Organic carbon concentration (mg/ml)	Dialyzed Soil Extract: Dilution to give minimum absorbance at 510 mμ	Dialyzed Soil Extract: Organic carbon concentration at dilution (mg/l)	Grams soil organic carbon/ dye equivalent
Mesa Verde				
1	2.0	600	3.4	170
2	0.8	300	2.7	130
76-2				
1	1.9	600	3.0	150
2	0.9	300	3.1	150

The final dye concentration was 2×10^{-5} M for these determinations.

be lengthened to increase the absorbance value. In this manner, we would estimate the sensitivity could be increased to 0.02-0.05 μ gm per ml. Another way to consider the sensitivity of the detection procedure is to extrapolate to the poorest soil with which we would expect to obtain a positive dye reaction using the standard extraction and dialysis procedure. The 76-2 soil has an organic carbon content of 0.4 percent. Of this organic carbon 8.5 percent can be extracted which is nondialyzable. This would give a concentration of 68 μgm/ml in a 20 percent (w/v) soil extract. Therefore assuming the same ratio of extractable nondialyzable organic carbon in soils with lower organic carbon content (which is probably a low value), a 70-fold lower organic carbon content would be detected on the basis of organic carbon at 1 μg/ml being detected by our present methods. This would suggest that an extract from a soil with an organic carbon content of 0.006 percent would give a positive result with the detection procedure.

In order to better evaluate the results obtained from the dye titration of humic acid it would be useful to know the concentration of the carboxyl groups on the humic acid. This was determined by titration of the carboxyl groups with a calcium acetate procedure (Blom et al., 1957). The choice of this system of determination was made on the basis of results reported by Wright and Schnitzer (1960). Their work reported the determination of carboxyl content of humic and fulvic acids by four procedures. These results showed that the calcium acetate procedures for the titration of carboxyl groups gave results compatible with an iodide-iodate procedure, but slightly lower than thermal decarboxylation and non-aqueous titration procedures. This lower value may result from steric considerations which prevent binding of the calcium ion. The calcium acetate procedure appeared to be the most readily implemented in our laboratory and it can be modified to reduce the sample required to acceptable limits. The most important requirement of the determination was that it indicate differences between samples from the same soil with a fair degree of accuracy and reproducibility. We found this to be the case with this procedure. Results obtained with this procedure are shown in Table XI. Variation of results with different samples from a single soil is seen in the fourth column (mequiv./gm). We interpret this to be a reflection of the degradation of the humic acid during preparation. In both cases shown the number 1 sample was more effectively protected from O_2 during the alkaline extraction and dialysis treatments. Exposure of humic acid to oxygen while in an alkaline solution is known to result in oxidation (Choudhri and Stevenson, 1957). The fifth column indicates the equivalent weight values for the carboxyl groups. The values shown for the number one sample in each case indicate a relatively small ratio of carboxyl groups per carbon atom. These values are considerably higher than the grams organic carbon per dye equivalent values indicated in Table X. The last column in Table XI directly compares the dye equivalent values with the carboxyl equivalents for the two preparations. The important point in this case is that the dye does not depend on the humic acid carboxyl

TABLE XI

DETERMINATION OF CARBOXYL CONCENTRATION IN DIALYZED SOIL EXTRACTS

Soil	Dialyzed Soil Extract			Soil organic carbon/carboxyl equivalent (g/equiv.)	Dye Equivalent*/ carboxyl equivalent
	Organic carbon concentration mg/ml	Carboxyl group concentration µequiv./ml	mequiv./gm organic carbon		
Mesa Verde					
1	2.0	2.3	1.1	910	5.4
2	0.8	3.8	4.8	210	1.6
76-2					
1	1.9	1.9	1.0	1000	6.7
2	0.9	2.2	2.4	420	2.8

* Values for dye equivalent employed for calculation of this column were obtained from Table X.

groups for its reactivity, but probably on the acidic phenolic groups. In the case of the Mesa Verde sample the carboxyl concentration can increase over 3-fold and there is only a slight increase in the dye reactivity (compare Mesa Verde samples 1 and 2 in Table X). In the case of the 76-2 soil the carboxyl concentration increases 2.4 times, but the dye reactivity is the same. However, analyses of a larger number of samples is required to make these conclusions more firm. Perhaps more importantly, these results do indicate the type of information that can be obtained with these experimental procedures.

Another approach to a determination of the effectiveness of the dye-humic acid carboxyl group reaction was to selectively decarboxylate the humic acid and determine what type of dye reaction the residue gives. This was to be accomplished by a procedure used to determine the carboxyl concentration of aromatic acids (Habacher, 1949) as applied to humic acids (Wright and Schnitzer, 1960) and involves heating the humic acid in quinoline in the presence of a copper catalyst. This appeared feasible since Schnitzer and Hoffman (1964) found phenolic groups to be more stable to pyrolysis than carboxyl groups. With selective catalytic decarboxylation it appeared that the carboxyl groups would be removed even more favorably than the phenolic groups. However, it was found that the decarboxylation treatment converted humic acid to a water insoluble form (partially soluble in organic solvents). Decreasing the conditions of decarboxylation from 250°C for 90 minutes to 200°C for 60 minutes still gave the same results. To determine if the change in solubility in water was due to the catalytic effect of the amine and copper salt, the humic acid was heated in mineral oil for 90 minutes at 250°C. This, also, converted the humic acid to an oil soluble, water insoluble material. It would appear that the phenolic groups are removed as rapidly as the carboxyl groups from the humic acid studied. Conversion of the humic acid to a water insoluble form makes it useless for studies with the thiacarbocyanine dye. Since other approaches looked more promising, further studies were not carried out to determine if still lower heating temperatures would have been effective in preferentially removing the carboxyl groups.

Further work to determine the nature of the dye-humic acid reaction involved acetylation and methylation. Methylation and acetylation of the humic acid can be carried out without apparent degradation of the humic acid and these two modifications offer a means of establishing the functional groups with which the dye reacts. Methylation of the humic acid was carried out in methanol with boron trifluoride etherate as catalyst. After isolation, the methylated humic acid was investigated with the dye. The methylation caused a small change in the dye spectrum in that the absorption maximum was shifted from 530 mμ to 550 mμ at the dye equivalence point (dye to humic acid ratio to give minimum absorption at 510 mμ). On the basis of dry weight, the methylated sample was only 1/2 as effective in changing the dye spectrum as the unmethylated humic acid. This result would appear

to be in conflict with the results obtained with the calcium acetate titration of humic acid carboxyl groups presented in a previous paragraph of this section. However, it is likely that the difference results from methylation of both carboxyl groups and acid phenolic groups and not methylation of just the carboxyl groups, which were shown by the carboxyl titration not to be too effective in their reaction with the dye. Comparison of the humic acid with the methylated humic acid on the basis of weight is not entirely satisfactory since no correction for the additional methyl groups is made, or for the possible presence of borate after the dialysis of the methylated mixture. Any such factor would give an apparent decrease in dye reactivity. That methylation did occur was shown by the changes in the infrared spectrum of the humic acid shown in Figure 17. The increase in absorbance at 5.8 microns and the decrease at 7.1 are indicative of ester formation. Evidence for methylation of acid phenolic groups is not as readily derived from the infrared data.

Results from an acetylation of humic acid are also shown in Figure 17. The changes are more dramatic than with the methylated sample. A peak forms at 5.8 microns and there is a marked decrease in absorbance at 7.1 microns. Acetylation of this sample was achieved with acetylchloride and tetrahydrofuran. No noticeable darkening of the humic acid occurred during the acetylation. A preliminary attempt was made to acetylate humic acid with an acetic anhydride and pyridine process employed by Wright and Schnitzer (1960). However, the conditions employed were found to be destructive as considerable darkening of the material occurred. Nevertheless, the milder acetylation with acetyl chloride in tetrahydrofuran gave a product which was insoluble in aqueous solution. This result does show that the hydroxyl and phenolic groups are important in the solubility of the humic acid. However, the insolubility of the acetylated product precluded any studies with the thiacarbocyanine dye. This suggests that this approach to studying the effect of hydroxyl and phenolic groups on the dye reaction would not be possible. There was not sufficient time to pursue the possibility of continuing this approach by partially acetylating the humic acid.

Included with the three infrared spectra of the 76-2 soil is a spectrum for the Mesa Verde soil. The main difference between the two soil extracts is the level of absorption over the 9-10 microns region with a maximum at roughly 9.5 microns. It is generally proposed that this absorption is due to the presence of silicate in the sample. There is roughly twice as much silicate in the 76-2 sample as in the Mesa Verde sample. Consequently, the difference in absorption between the two samples is in the right direction.

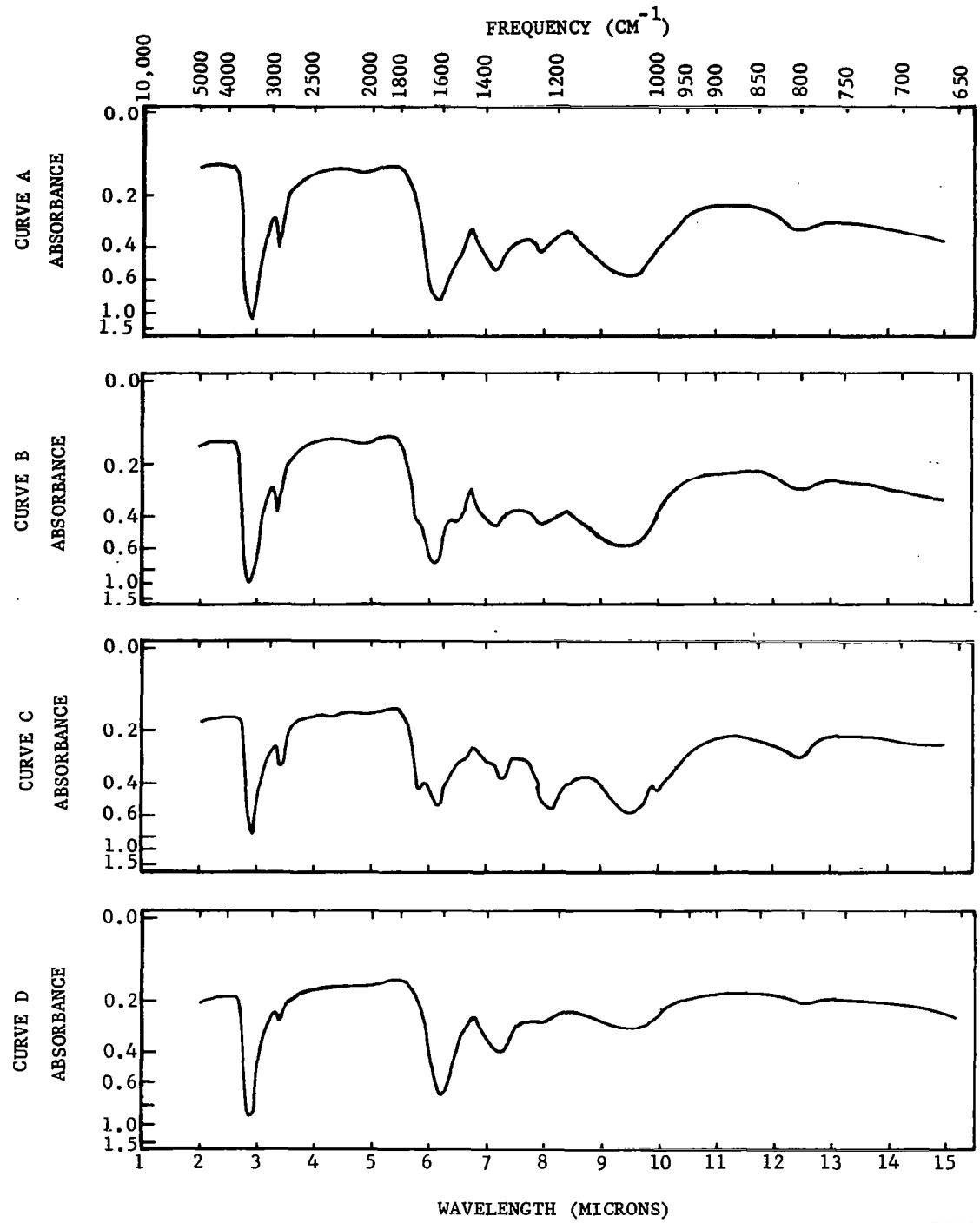

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FIGURE 17. INFRARED SPECTRA OF HUMIC ACID.

Spectrum A is for the dialyzed extract of soil 76-2. Spectrum B is for the methylated product of the extract from soil 76-2. Spectrum C is for the acetylated product of the soil extract from soil 76-2. Spectrum D is for the dialyzed Mesa Verde soil extract. Spectra were determined on KBr pellets.

SECTION 4

CONCLUSIONS

Research on the interaction of biological macromolecules with the dye, 4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine as a highly sensitive method for use in life detection experiments has reached the stage where discussion of its readiness for application is desirable. We believe the utility of the system as a means of detecting macromolecules has been established and an effort should be directed toward instrumentation of the dye system.

The areas of research which this study of the dye detection system covered included: (a) demonstration of the sensitivity of the dye to organic polymeric substances in soil extracts, pure compounds and mixtures of pure compounds, (b) accumulation of data on the dye reaction with a partial elucidation of the reaction mechanism and (c) the delineation of materials which can interfere with the detection scheme and ways to circumvent this interference.

Work required to cover these areas included such diverse studies as humic acid extraction and fractionation, composition of humic acid, mechanism of dye reaction with macromolecules, the fate of inorganic polyanions in aqueous solution, rates of dialysis, and depolymerization of aluminosilicate minerals. The information obtained permitted a decision to be made as to how the system could best be employed as an individual experiment or used to supplement other detection experiments. The dye system can be implemented as an individual experiment for detecting polyanionic polymers, or it can be used for this purpose in an integrated experiment, such as the Automated Biological Laboratory. As an individual experiment, it would have great utility as an extremely sensitive screening system for organic polymers in soil samples and can be simplified to comply with the

strict weight requirements of space exploration. As part of an integrated package or in more complex form as an independent experiment, it can be employed effectively for screening soil samples, polymer analysis and recognition of polymer replication (as a growth indicator). For polymer analysis, fractionation of the polymeric material will permit the dye system to be used for determining the spatial arrangement of anionic functional groups in biological polymers. The system has many advantages including sensitivity of detection, simplicity of data readout, amount of information which can be derived from the spectral changes induced by macromolecules, and the wide concentration range over which the polymeric material can vary without overloading the detection system. The requirements of the system include that the dye solution have an absorbance on the order of one (e.g., with a one centimeter light path, the dye must be at 1×10^{-5}M), the concentration of inorganic polymers must be low relative to the organic polymer, the test solution should be free of small molecular weight anionic materials.

SECTION 5

RECOMMENDATIONS

Recommendations for further research on the detection procedures include: (a) instrumentation, (b) desert soil research, and (c) comparison of dye detection procedure with other appropriate detection procedures for sensitivity and applicability. Development of instrumentation for the experiment will require the specific application of wet processing techniques, developed at Aeronutronic, to the extraction, fractionation (if desired) and dialysis requirements of the detection system. Some preliminary work was presented previously in an Aeronutronic proposal. However, this work requires updating because of subsequent findings in research on the detection method. More research on desert soil is required because of the limited information available concerning organic constituents of desert soils, their size distribution and characteristics. This work will be valuable to the Mars missions in any event, but will be specifically important to describing the limits of sensitivity of the dye detection system. As mentioned in Section 3, no specific effort was made to determine the ultimate sensitivity of the dye detection system. Nevertheless, the calculation was made that a 1.0 μg. organic carbon/ml. concentration of soil humic acid could be detected with a one-cm. light path, or that the lowest organic carbon content of a soil which would give a positive dye test would be 0.006 percent. This latter value required the assumption that the percent of nondialyzable organic compounds in soils of low organic carbon content (i.e., <0.4 percent) does not decrease. In addition, this value of 0.006 percent organic carbon could be decreased at least 10-fold by modifications of the sample holder and instrument design. As an example, the cross section of the solution container for the spectrophotometer could be substantially reduced decreasing the amount of solution required and, consequently, the amount of test material required for a positive test. However, these extrapolations should be confirmed by experimental work on desert soils of very low organic carbon content.

The last area of study proposed is an experimental comparison of other procedures and techniques with the dye detection method for their applicability and versatility to the problem of detecting biological macromolecules in soil. Such possibilities as methylation or acetylation of biological macromolecules with radioactively labeled or fluorescent labeled materials or the use of infrared absorption or fluorescence should be considered.

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