THE ULTRAVIOLET ABSORPTION SPECTRA OF DESOXYPENTOSE NUCLEIC ACID*

by

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Since the discovery that nucleic acids, and more particularly the purine and pyrimidine components thereof, were mainly responsible for the strong ultraviolet radiation absorbing properties of cells[1], several reports have appeared on the ultraviolet absorption spectra of nucleic acids, nucleotides, nucleosides, purines, and pyrimidines isolatable from tissues[2,3,4,5]. There have also been attempts to correlate quantitatively the ultraviolet absorption of cells and cellular domains with the nucleic acids and protein components of such areas[6]. To do this, it is obviously necessary to know (1) the absorbance of such substances per unit of concentration; (2) whether the absorption of radiation by such substances varies normally with the concentration and thickness of the substances, *i.e.*, whether they obey the BOUGUER-BEER-LAMBERT Law; and (3) whether the absorbance values of the substances are influenced by their chemical environment. In this paper we consider the ultraviolet absorption of desoxypentose nucleic acid with regard to these factors and with respect to its macromolecular structure.

EXPERIMENTAL

Materials: The sodium desoxyribonucleate was a fibrous high molecular weight sample prepared from calf thymus by Dr. D. O. JORDAN and was kindly supplied by him along with the following elementary analysis: C, 38.7%; H, 3.95%; N, 15.8%; P (colorimetric) 9.29%. This material was prepared according to the method described by GULLAND, JORDAN AND THRELFALL[8]. The loss of weight upon drying at 110° C *in vacuo* over P_2O_5 was 21.4%. Our phosphorus determinations were made on aqueous solutions by a modification of SPERRY's method[15].

As may be seen from the data presented in the figures, SDN in aqueous solution at pH 6.6 has an absorptivity (k) at 259 mμ (λmax.) of 25.5 and at 233 mμ (λ min.) $k = 9.5$. The $A^{1\%}_{1cm}$ at 259 mμ is 258, ε(P)[16] is 8500. Our value for $A^{1\%}_{1cm}$ (259 mμ) is considerably higher than data previously reported by others. SDN in 1 M NaCl at pH 6.8 at 259 mμ has $k = 21.7$, and $A^{1\%}_{1cm} = 213$.

The lysozyme was a generous gift from Dr. L. L. UZMAN who prepared it from hen egg white. It was a five-times crystallized sample obtained by an initial seeding of the egg white at the isoelectric point with lysozyme prepared according to FEVOLD AND ALDERTON[17], and repeating the isoelectric crystallization four times. The five-times crystallized lysozyme thus obtained was electrophoretically and ultracentrifugally homogeneous.

The bovine plasma albumin used was crystalline material obtained from Armour and Company and contained less than 1% salt.

The polyvinyl alcohol was a commercial sample (du Pont grade 91-65) which is a high viscosity material prepared by the polymerization of vinyl acetate followed by hydrolysis of more than 98.5%

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of the acetate groups. It was dialyzed against distilled water (several changes) to remove any residual salts.

All solutions were made using glass distilled water.

Apparatus: Unless otherwise specified, all spectral absorption measurements were made using a Cary recording spectrophotometer and matched fused quartz absorption cells. The uncertainty in the measurements is no greater than ± 0.015 units at absorbances of 1.3 and no greater than ± 0.03 units from 1.30 to 2.90.

A Beckman spectrophotometer and short path length cells consisting of 10 mm quartz cells and quartz "stuffers" of appropriate length were used in some of the measurements reported in Figs. 3 and 4.

All pH measurements were made using the glass electrodes of a Beckman Model G pH meter.

RESULTS

In order to determine accurately (by ultraviolet light absorption measurements) the amount of nucleic acid in the mélange of materials that exist in a cell, it is certainly necessary to know the absorptivity* (specific absorbance or absorbance per unit concentration per unit path length) of the pure substance. Since the molecular weights of desoxypentose nucleic acid (SDN)** samples vary depending on the preparation and other factors, and since it is very difficult to weigh SDN accurately because of its hygroscopic nature, it does not appear feasible to determine the molar absorptivity or molecular extinction coefficient of SDN. However, knowing the weight concentration of the absorbing substance, it is possible to determine the absorptivity. The concentration is best obtained by determining the amount of phosphorus per ml of an aqueous SDN solution with the knowledge of the phosphorus content of pure SDN[8]. Thus knowing concentration, path length, and absorbance ($\log_{10} I_0/I$) of a solution, we make use of the relation: absorptivity equals absorbance divided by concentration in grams per liter times path length in cm, or

$$k = \frac{A}{c \cdot d},$$

and plot the absorptivity (k) of SDN solutions (Fig. 1) as a function of wavelength.

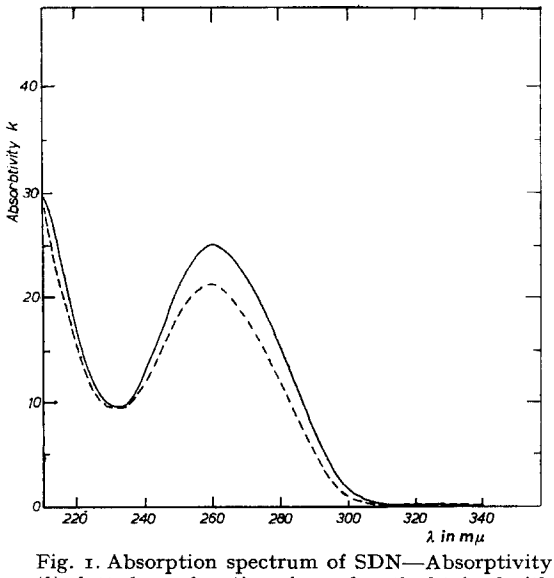

Fig. 1. Absorption spectrum of SDN—Absorptivity (k) plotted as a function of wavelength obtained with 0.00446% SDN solutions
——— in water pH 6.7; – – – – in 1 *M* NaCl pH 6.8.

An examination of whether the ultraviolet absorption of SDN is directly proportional to concentration and thickness is especially necessary because of the variations in these factors met in ultraviolet absorption microspectroscopy of cells[9]. Owing to the difficulties in making and accurately measuring the thickness of absorption cells having path lengths of 1 μ to 50 μ, we have not yet examined SDN solutions in the high concentrations and short path lengths found in tissues, although such measurements are contemplated because of their obvious utility for

* We use here the terminology of BRODE[7].

** Hereafter designated as SDN, meaning the sodium salt of desoxypentose nucleic acid.

correlation with ultraviolet absorption microspectroscopy of tissues. However, a range of concentrations and path lengths of aqueous SDN solutions amenable to measurement by a Cary Model 11 recording spectrophotometer and a Beckman DU spectrophotometer have been examined. Fig. 2 presents results showing that, at least for concentrations as great as about 0.004%, the absorbance at 259 mμ of aqueous solutions of SDN is directly proportional to path length.

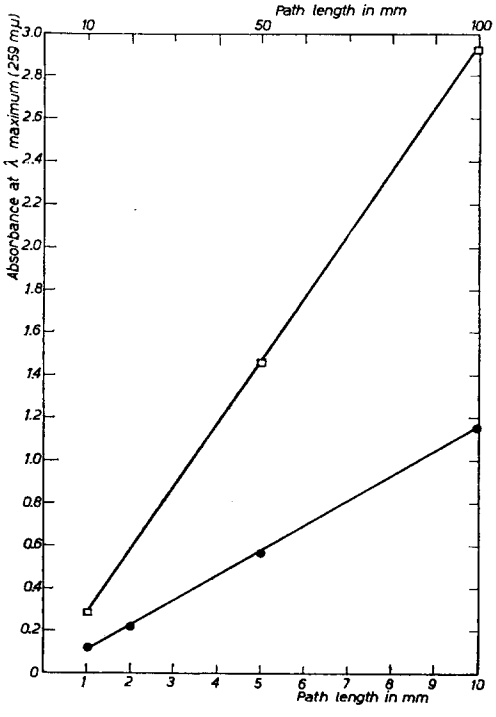

Fig. 2. Absorbance at 259 mμ as a function of path length for aqueous SDN
□——□ concentration 0.00115% SDN (Cary data)
●——● concentration 0.00446% SDN (Beckman data)
The path lengths for the low concentration are given in the upper abscissa scale, the path lengths for the higher concentration are on the lower abscissa scale.

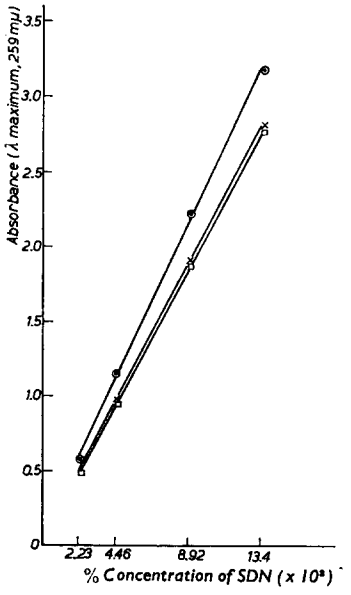

Fig. 3. Absorbance at 259 mμ as a function of concentration for
⊙——⊙ SDN in distilled water
X——X SDN in 0.1 *M* NaCl solution
⊡——⊡ SDN in 1.0 *M* NaCl solution

Figs. 3 and 4 show the relationship between absorbance and concentration. From Fig. 3 it may be seen that for concentrations not exceeding about 0.01% the 259 mμ absorbance is proportional to the concentration, indicating compliance with the BOUGUER-BEER-LAMBERT Law. Fig. 4, however, shows that at higher concentrations the solutions no longer conform to the ideal relationship. No data were obtained for concentrations exceeding 0.044% because, even using the shortest solution cells at hand, the absorbance became too great to measure reliably. As the concentration is increased beyond 0.01%, and with path length decreased inversely so as to keep the product of concentration and path length constant, the absorbance begins to decrease; doubling the concentration (and halving the path length) decreases the absorbance by about 3%.

Since SDN does not exist *in vivo* in a pure water solution, it is pertinent to examine

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the effect upon the absorption of SDN by substances that are present in quantity in

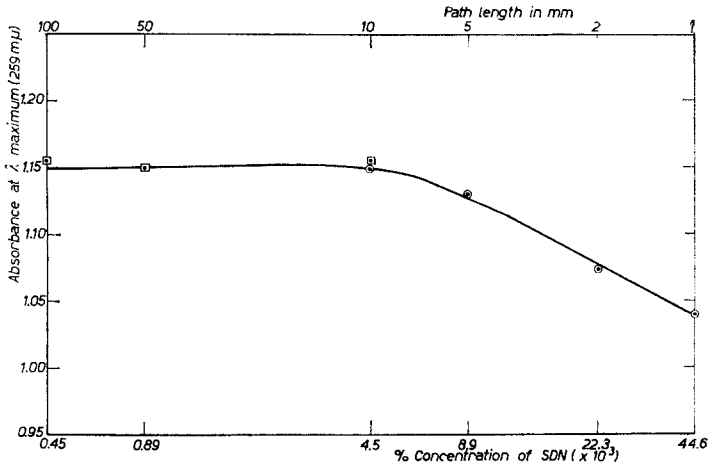

Fig. 4. Absorbance at 259 mμ as a function of path length and concentration for aqueous SDN solutions. Cary data [·] Beckman data ◉

tissues, *e.g.*, inorganic salts and proteins. The addition of sodium chloride to aqueous solutions of SDN lowers the absorbance (between pH 6.5 and 7.0) by an amount varying linearly with the logarithm of the salt concentration. Data for four different concentrations of SDN are shown in Fig. 5, where it may be seen that concentrations of salt around 10^{-3} *M* will decrease the absorbance of aqueous SDN about 10%, relative to an SDN solution without added salt, whereas salt concentrations around 1 *M* will decrease the absorbance as much as 17%. The magnitude of this effect makes it apparent that measurements of concentration of SDN solutions to accuracies

TABLE I

0.00446% SDN aqueous solution with following substances added	*pH of solution*	*Absorbance at λmax. (259 mμ) Path length = 1 cm*
None	6.7	1.15
H_3BO_3 0.4 *M*	4.6	1.23
Phosphate buffer	7.9	0.92
(Na_2HPO_4 + KH_2PO_4)	7.02	0.95
	5.95	0.92
Urea 5 *M*	7.9	1.06
Urea 2.5 *M*	7.6	1.09
Urea 0.5 *M*	7.4	1.12
Glycine 1 *M*	6.3	1.02
L-asparagine 1%	4.8	1.15
Glycerine (60% solution)	6.7	1.14
Sucrose (20% solution)	7.4	1.12

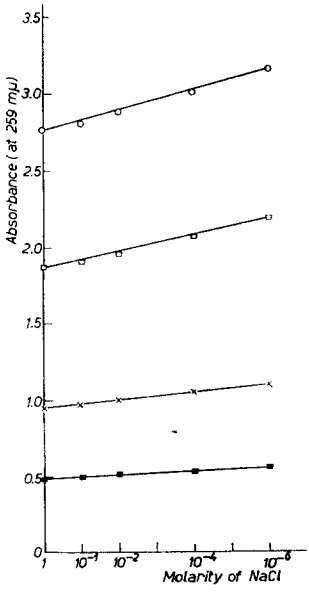

Fig. 5. Absorbance at 259 mμ as function of sodium chloride concentration for four SDN concentrations

○ = 0.0134 % SDN;
□ = 0.00892 % SDN;
× = 0.00446 % SDN;
■ = 0.00223 % SDN

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of greater than ± 10% can be made only if the salt concentration in the solution is known.

Solutions of other salts such as sodium phosphate (phosphate buffer at pH 7.0) show similar effects on the absorbance of aqueous SDN solutions. Likewise the effect of glycine is to lower the absorbance of SDN solutions. On the other hand, large concentrations of relatively nonionic substances such as sucrose or glycerine do not materially affect the ultraviolet absorption of SDN solutions. Their results are summarized in Table I.

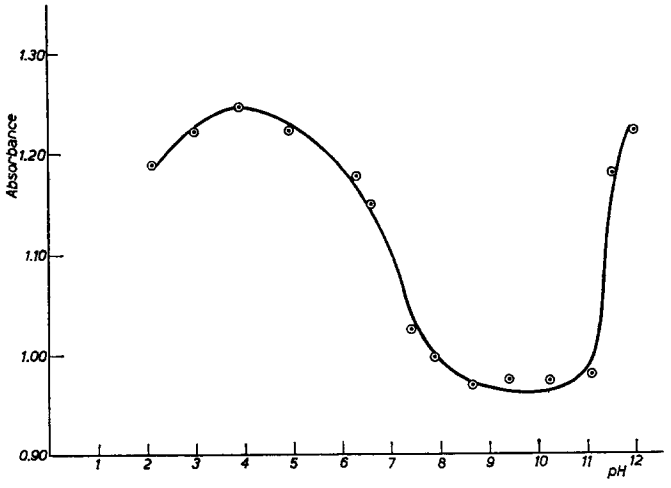

Fig. 6. Absorbance at 259 mμ as a function of pH for 0.00446 % SDN in distilled water.

The effect of pH on the intensity of absorption of aqueous solutions of SDN, without added salt, is seen in Fig. 6. The maximum absorption occurs at pH values less than 6 and greater than 11 in direct contrast to the earlier work of FRICK[10], who used salt buffered solutions and found practically unchanging absorbance between pH 3 and pH 10. It is also interesting to note that all SDN solutions having pH values less than 10 seem to have an isosbestic point at 233 mμ where the absorptivity is 9.5 ± 0.2 (Fig. 7) and $A^{1\%}_{1\,cm}$ is 96*. Since the absorbance at this wavelength is pH

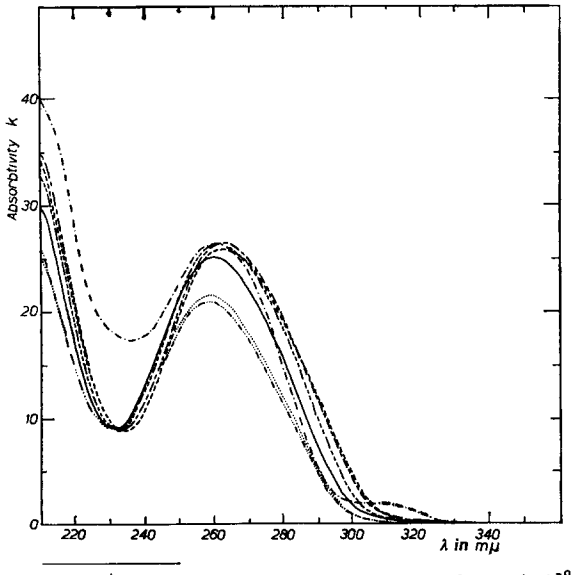

Fig. 7. Absorption spectra of 0.00446 % aqueous SDN solutions at various pH values (unbuffered solution). ------- pH 2.1; ——— pH 3.1; ---------- pH 4.9; ———— pH 6.7; ····· pH 7.9; ···—···— pH 10.3; —·—·— pH 12.0

* It should be emphasized that values of $A^{1\%}_{1\,cm}$ given here and in other papers are only valid if computed from dilute solution measurements, since at concentrations greater than $4.5 \cdot 10^{-3}$ % SDN does not obey BEER's Law (cf. Fig. 4).

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independent in the pH range 2 to 10, it is suggested that measurements at this wavelength may be useful for assessing purity of nucleic acid preparations.

The effect of certain proteins upon the ultraviolet absorption of aqueous SDN solutions has been studied. Five- to forty-fold excess of protein to SDN was used since this is the approximate ratio of the two types of material which occur in tissues. Addition of aqueous protein solutions to aqueous SDN solutions in some cases produced precipitation and/or opalescence even in dilute solutions. This, in combination with the inherent ultraviolet absorption of proteins, due to their aromatic amino acid content, makes ultraviolet absorption measurements of such solutions difficult. The presence of one molar sodium chloride reduces the tendency to precipitation and ultraviolet measurements have been made on such solutions. Typical absorption spectra are shown in Fig. 8 and data in Table II.

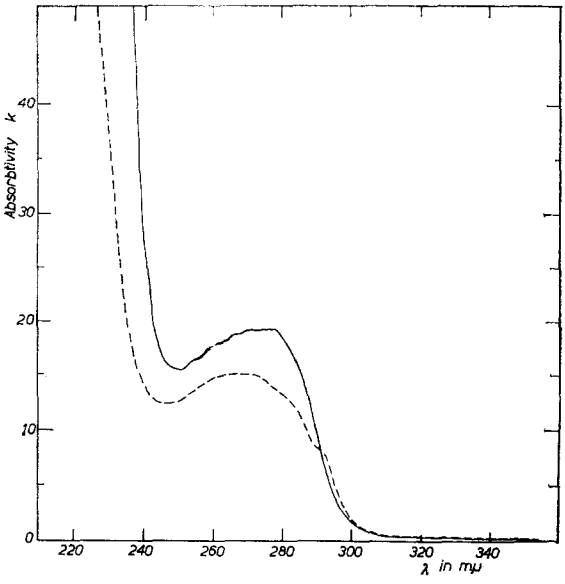

Fig. 8. Absorptivity as a function of wavelength for 0.00223% SDN in 1 *M* NaCl in the presence of ——— 0.0937% plasma albumin and – – – – 0.0205% lysozyme. The measurements were made using a water blank.

It may be seen from the foregoing table that the presence of lysozyme or plasma albumin in aqueous solutions of SDN, in general, tends to decrease the absorption by the nucleic acid moiety. In the presence of salt, the effect is even more pronounced as the absorbance is generally lower for such solutions than for equivalent amounts of SDN and salt in the absence of the proteins. Scattering and diffusion of the incident radiation as well as deviations from the BOUGUER-BEER-LAMBERT Law will

TABLE II

*Solution**	*pH*	*λmax. in mμ*	*Absorbance*
0.00223% SDN	6.3	259	0.58
0.00446% SDN	6.3	259	1.17
0.00223% SDN + 0.0937% Bovine plasma albumin (PA)	6.17	259	0.47
0.00223% SDN + 0.0937% PA in 1 *M* NaCl	5.9	259	0.47
0.00223% SDN + 0.04685% PA	6.53	259	0.47
0.00223% SDN + 0.04685% PA in 1 *M* NaCl	6.1	259	0.47
0.00446% SDN + 0.04685% PA	6.85	259	0.98
0.00446% SDN + 0.04685% PA in 1 *M* NaCl	6.2	259	0.94
0.00223% SDN + 0.0205% lysozyme in water		Precipitate	
0.00223% SDN + 0.0205% lysozyme in 1 *M* NaCl	5.3	260	0.45
0.00446% SDN + 0.0205% lysozyme in water		Precipitate	
0.00446% SDN + 0.0205% lysozyme in 1 *M* NaCl	5.1	260	0.93

* In each case the solution spectra were determined using the appropriate aqueous solution of protein and salt as a blank so that the "difference spectra", that of SDN, was obtained.

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have an important effect on such absorption measurements, and this may well explain some of the minor differences in Table II.

DISCUSSION

Several conclusions may be drawn from the foregoing results. First, that the *intensity* of absorption of SDN solutions is dependent on the ionic strength. This is clearly shown by the many determinations using sodium chloride (Fig. 5) in which concentrations as low as 10^{-5} molar in sodium chloride significantly lowered the intensity of absorption of SDN solutions. Further, it was observed that addition of other ionizable substances such as boric acid and phosphate buffer had an analogous effect, whereas the addition of glycine had a similar but smaller effect on the intensity of absorption. Secondly, our data indicate that the intensity of absorption of aqueous SDN is highly pH-dependent, in the absence of salt, over the pH range 3 to 12 (Fig. 6), and that the intensity of absorption is practically the same at the two extremes of pH.

These two facts make it evident that ionized forms of nucleic acid are large contributors to its ultraviolet absorption. It may be further argued that the ionized phosphate groups of nucleic acid probably are not direct contributors to the ultraviolet absorption since the *absorption is high both in acidic and basic solutions.* In addition, the phosphate groups are well removed from the purine and pyrimidine bases by the sugar residues which themselves show no radiation absorption around 260 mμ. This prompts consideration of the effect of pH and ionic strength on the purine and pyrimidine components. The ultraviolet absorption of these compounds[11,12,13] has been measured in aqueous solution at acidic, neutral and basic pH's, and although the ultraviolet absorption of solutions of these materials is highly pH-dependent, no unique effect of pH change upon the absorption intensity of the free bases has been observed. Furthermore, when the ultraviolet absorption of an approximately equimolar aqueous solution of thymine, cytosine, adenine and guanine is measured and compared with the same solution containing 1 *M* sodium chloride, no significant difference is observed (Table III). Similar results were obtained with a mixture of ribonucleotides.

TABLE III

Solution	*pH*	*λmax. in mμ*	*Absorbance*
1. Mixture containing $3.7 \cdot 10^{-5}$ *M* adenine, $3.3 \cdot 10^{-5}$ *M* guanine, $4.5 \cdot 10^{-5}$ *M* cytosine and $3.4 \cdot 10^{-5}$ *M* thymine in water	6.6	263	1.25
2. Same mixture as (1) in 1 *M* NaCl	6.8	263	1.26
3. Mixture containing ribonucleotides: $8.6 \cdot 10^{-5}$ *M* adenylic acid, $1.6 \cdot 10^{-4}$ *M* guanylic acid, $1.5 \cdot 10^{-4}$ *M* cytidylic acid, $1.5 \cdot 10^{-4}$ *M* uridylic acid	4.5	258	1.40
4. Same mixture as (3) in 1 *M* NaCl	4.7	258	1.43
5. 0.00446% SDN + 0.02% polyvinyl alcohol in water	6.8	259	1.11

We are therefore led to the conclusion that the ultraviolet absorption of SDN is not simply the sum of its individual constituents, but rather reflects the interaction that must occur between the component purines and pyrimidines when bound in nucleic acid molecules. We may visualize the action of salt as effectively separating the ab-

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sorbing groups from each other and thus lowering the absorption, whereas changing the pH to acid or alkaline places charges on the purines and pyrimidines, makes their electrostatic interaction more likely, and thus increases the intensity of absorption.

If we now consider the effect of some high molecular weight compounds on the ultraviolet absorption of aqueous SDN solutions, we note that addition of lysozyme, which has an isoelectric point between 10.5 and 11[14], results in opalescence and precipitation even in relatively low concentrations of lysozyme probably because of the formation of a highly polar complex between the SDN and the lysozyme. The presence of molar sodium chloride (Table II) prevents the precipitation, and measurements of ultraviolet absorption indicate that although the salt effect predominates, the intensity is lowered still further by the lysozyme.

The addition of bovine plasma albumin, which has an isoelectric point around 5.5, does not result in precipitation when added to dilute solutions of SDN. However, in each case (Table II), whether in the presence of added sodium chloride or not, the addition of plasma albumin results in a lowering of the intensity of ultraviolet absorption of the SDN. This result probably can be interpreted similarly to the salt effect described above, that is, the association of the charged groups on the surface of the albumin molecules with the purines and pyrimidines of the SDN prevents their association with each other and thus lowers the absorption intensity.

Thus, we seem to be dealing with three effects: (1) a pH effect which tends to increase absorption intensity at high and low pH's probably due to interaction of neighbouring absorbing units in nucleic acid molecules, (2) a "small ion" effect which decreases this tendency to associate as evidenced by lowered absorption upon the addition of salts, (3) a "macromolecular ion" effect which also lowers absorption of SDN solutions.

Further evidence that these are ionic effects is seen in the addition of both small molecule and macromolecular non-ionic substances such as sucrose and glycerol (Table I) and polyvinyl alcohol (Table III), all of which seem to have little effect on the absorption intensity of aqueous SDN solutions even when the non-ionic molecules are present in very large quantity.

From the foregoing it is evident that care must be exercised in using measurements of absorption intensities in the ultraviolet for determination of the nucleic acid content of tissues and cells or extracts thereof, unless the ionic strength and the pH are known. It is of course apparent, too, that this applies to measurements reported during the past few years for the purpose of assessing purity of particular nucleic acid preparations.

SUMMARY

The intensity of the ultraviolet absorption of sodium desoxypentose nucleate (SDN) in aqueous solution is lowered by the addition of salts, such as alkali halides and phosphates. SDN absorbance in sodium chloride solutions has been carefully measured and appears to vary linearly with the log of the sodium chloride concentration over a 10^5 change in salt concentration. In the absence of salt, the ultraviolet absorption of SDN solutions show strong pH dependence between pH 3 and pH 11. Aqueous SDN at pH 6.7 in the absence of added salt has an absorptivity (k) of 25.5 and $A^{1\%}_{1\,cm}$ of 258 at 259 mμ (when measurements are made on concentrations less than 0.01%). These values are considerably higher than previously reported. Aqueous SDN solutions in the pH range 2 to 10 have an isosbestic point at 233 mμ.

Addition of lysozyme and bovine plasma albumin to SDN solutions also result in a decrease in the ultraviolet absorption due to SDN. On the other hand, the addition of non-ionic substances such as polyvinyl alcohol, sucrose, or glycerol to aqueous SDN solutions, even in high concentrations,

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does not decrease its ultraviolet absorption. An explanation of these data in terms of the association of the component purine and pyrimidine groups is suggested.

These results indicate that caution must be exercised in using measurements of absorption intensities in the ultraviolet for the determination of the purity of nucleic acid preparations and for the determination of the nucleic acid content of tissues and cells unless the ionic strength, pH, and BEER's Law deviation are known.

RÉSUMÉ

L'addition de sels tels que des halogénures et des phosphates alcalins à des solutions aqueuses de désoxypentose nucléate de sodium (SDN) diminue l'intensité de leurs absorptions en U.V. Le coefficient d'absorption du SDN dans des solutions de chlorure de sodium a été mesuré avec soin et varie linéairement avec le logarithme de la concentration en chlorure de sodium quand cette concentration est multipliée par 10^5. En l'absence de sel, l'absorption U.V. de solutions de SDN dépend étroitement du pH entre pH 3 et pH 11. Une solution aqueuse de SDN à pH 6.7 en l'absence de sels, a un coefficient d'absorption (k) de 25.5 et un $A^{1\%}_{1\,cm}$ de 258 à 259 mμ (les mesures étant faites sur des solutions dont les concentrations sont inférieures à 0.01 %). Ces valeurs sont nettement plus élevées que celles données antérieurement. Les courbes d'absorption de solutions aqueuses de SDN, déterminées à des pH compris entre 2 et 10, se coupent à 233 mμ ("isosbestic point").

L'addition de lysozyme ou de sérumalbumine de boeuf à des solutions de SDN entraine également une diminution de l'absorption U.V. du SDN. Au contraire l'addition, même à des concentrations élevées, de substances non ionisables telles que l'alcool polyvinylique, le sucrose ou le glycérol à des solutions aqueuses de SDN ne diminue pas l'absorption U.V. Ces résultats s'expliquent peut-être en fonction de l'association des groupes puriques et pyrimidiques.

Ils montrent que la détermination de la pureté de préparations d'acides nucléiques et la détermination de la teneur en acides nucléiques des tissus et des cellules à partir des intensités d'absorption en U.V. doivent être faites avec prudence, à moins que la force ionique, le pH et l'écart avec la loi de BEER ne soient connus.

ZUSAMMENFASSUNG

Die Intensität der Absorption von Natriumdesoxypentosenucleat (SDN) im Ultravioletten in wässriger Lösung wird durch den Zusatz von Salzen wie Alkalihalogeniden und -phosphaten vermindert. Die Absorption der SDN in Natriumchloridlösungen wurde sorgfältig gemessen und scheint von dem Logarithmus der Natriumchloridkonzentration über eine 10^5 Veränderung der Salzkonzentration linear abhängig zu sein. Bei Abwesenheit von Salz zeigt die Absorption der SDN-Lösungen im Ultravioletten eine strenge Abhängigkeit vom pH zwischen pH 3 und pH 11. Ohne hinzugefügtes Salz besitzt die wässrige SDN-Lösung bei pH 6.7 eine Absorption (k) von 25.5 und $A^{1\%}_{1\,cm}$ von 258 bei 259 mμ, wenn die Messungen bei Konzentrationen, die niedriger als 0.01 % sind durchgeführt werden. Diese Werte sind beträchtlich höher als die früher berichteten. Wässrige SDN-Lösungen im pH-Bereich von 2–10 besitzen einen isosbestischen Punkt bei 233 mμ.

Der Zusatz von Lysozym und Rinderplasmaalbumin zu SDN-Lösungen ergibt ebenfalls eine der SDN zuzuschreibende Verminderung der Absorption im Ultravioletten. Auf der anderen Seite vermindert der Zusatz von nichtionischen Substanzen wie Polyvinylalkohol, Rohrzucker oder Glyzerin zu wässrigen SDN-Lösungen sogar in hohen Konzentrationen die Absorption im Ultravioletten nicht. Eine Erklärung dieser Tatsachen wird in der Assoziation der Purin- und Pyrimidingruppen vermutet.

Diese Ergebnisse zeigen, dass man Vorsicht walten lassen muss bei der Benützung von Messungen der Absorptionsintensität im Ultravioletten bei der Bestimmung der Reinheit von Nucleinsäurepräparaten und bei der Bestimmung des Nucleinsäuregehaltes von Geweben und Zellen, wenn nicht die Ionenstärke, das pH und die Abweichung vom BEER'schen Gesetz bekannt sind.

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