A sample of octapeptide-acid of lesser purity (m.p. 180–187°) gave on reduction a yield (based on biological activity) of 38%.

A sample containing 510,000 pressor units and 16.0 mg. of nitrogen (32,000 u./mg. N) in methanol and HCl was neutralized by the addition of 0.33 ml. of 1 *N* NaOH and concentrated *in vacuo* to a small volume. The *p*H was then adjusted to 1.4 and the solution equilibrated with the upper phase of the system *n*-butanol–*n*-propanol–0.1 *N* HCl 1:1:2 (*p*H 1.4).

Both phases were then added to the first 4 tubes of a 100 tube countercurrent apparatus. The octa-peptide was distributed for 330 transfers by the single withdrawal method. The samples that came off starting with transfer 97 were combined into batches of 10. To each batch was added 1/2 volume of petroleum ether, and the activity was extracted into 3 times 40 ml. of 0.1 *N* HCl. The *p*H of the combined extracts was adjusted to 2.5–3 and the solution concentrated *in vacuo* to 10 ml. Each sample was assayed for pressor activity and nitrogen content.

Two nitrogen peaks were obtained: the first one (maximum in tube with transfer no. 160, K = 1.54) contained 25% of the total nitrogen and 8% of the pressor activity. The second peak (maximum in tube no. 230, K = 0.73) contained 59% of the total nitrogen and 83% of the pressor activity. The specific activity of the middle samples of the second peak was 55,000 pressor u./mg. N.

One single spot was obtained on paper chromatography in two solvent systems, R_f (BAW) = 0.25, R_f (MPW) = 0.44.[24] On hydrolysis and two-dimensional paper chromatography of the hydrolyzate 8 ninhydrin-positive spots were obtained which were identified from their R_f as aspartic acid, arginine, valine, tyrosine, isoleucine, histidine, proline and phenylalanine. They were found to be present in about equimolar amounts when compared to known mixture of the same amino acids ran simultaneously. The distribution coefficient of the octapeptide was determined in the following 2 solvent systems: (a) *n*-butanol–0.01 *N* Na_2CO_3–$NaHCO_3$ buffer K = 0.21; (b) *sec*-butyl alcohol-methanol–0.5% NaCl solution 19:1:20, *p*H adjusted with concd. NH_3 to 9.6, K = 0.40.

(24) Samples applied to the paper without desalting.

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Deformation of Deoxyribonucleate. II. Precipitation of Heat-deformed DNA with Millimolar Lead Ion[1]

BY VINCENT L. STEVENS AND EDWARD L. DUGGAN

RECEIVED APRIL 8, 1957

Heat-deformed deoxyribonucleate (DNA) may be separated from unheated DNA with lead ion. Deformation of secondary structure of DNA due to mild heating changes the unique physico-chemical properties of this substance. This report is concerned with the observation that lead ion forms an insoluble precipitate with DNA which has been heated above 87°. DNA unheated forms no precipitate. This observation suggests new possibilities for DNA purification and assay of purity, in terms of deformed DNA *versus* isolated DNA.

Certain properties of the nucleic acids (RNA and DNA) result from their high negative charge. As phosphoryl polymers having an average residue weight of 312, one negative charge should reside on each phosphoryl group at all *p*H values in the physiological range. One could consider the material to be related in this respect to polymers of acrylic acid or to the low cross-linked sulfonated polystyrenes. These natural macro-ions should react to accumulate various cations, when they exist in the free state, uncombined to proteins.

On this basis, it is perhaps not too surprising that DNA is maintained in an intact form only by careful control of the enzymatic, thermal and ionic history of each preparation. Reports from this Laboratory,[2] as well as the work of Thomas,[3] Doty,[4] Cox and Peacocke[5] and Cavalieri, *et al.*,[6,7] demonstrate the importance of solution conditions, and the relative stability of DNA samples at high ionic strength. These effects are independent of the particular cations present (Na, K, Mg) if sufficient ionic strength is maintained.

Any effect of a specific metal ion upon DNA should be assessed in an ionic environment such that the contribution of this metal ion to the ionic strength is insignificant. Shack and co-workers[8] have investigated the effect of certain alkali and alkaline earth cations upon the absorbance of DNA at 260 mμ. Their results stem from work with DNA samples which may have been exposed to ion-lack by storage in water, and their study included a wide variation of ionic strength during the experiments.

Inagaki[9] found that lead ion would precipitate nucleotides at neutral *p*H. Others[10] observed delayed metal sulfide precipitation in the presence of DNA. Lead ion interaction with DNA was investigated in this Laboratory, as a possible means of estimation of terminal phosphate groups on DNA. Lead ion was chosen for study on the basis that insoluble $PbHPO_4$ occurs, the combination with hydroxide ion may be ignored at *p*H 3–5, and lead ammine complexes are uncommon. The preliminary report[1] indicated that *apparent* proton replacement by lead ion was such that 1 in each 12 was a terminal phosphorus. This value seemed incompatible with the accepted molecular weight of the substance, unless branching exists. Values

(1) Abstracted from the thesis to be presented by V. L. Stevens for the Ph.D. degree, 1957. The work was supported by U. S. P. H. Grant C-2287 (C4).

(2) E. L. Duggan and V. L. Stevens, *Federation Proc.*, **14**, 205 (1955); **15**, 245 (1956).

(3) R. Thomas, *Biochim. Biophys. Acta*, **14**, 231 (1954).

(4) P. Doty, abstracts, A. C. S. meeting, Dallas, Texas, April 8–13, 1956, p. 17-Q.

(5) R. A. Cox and A. R. Peacocke, *J. Chem. Soc.*, 2499 (1956).

(6) L. F. Cavalieri and B. H. Rosenberg, *Biochim. Biophys. Acta*, **21**, 203 (1956).

(7) L. F. Cavalieri, B. H. Rosenberg and M. Rosoff, THIS JOURNAL, **78**, 5235, 5239 (1956).

(8) J. Shack and J. M. Thompsett, *J. Biol. Chem.*, **197**, 17 (1952).

(9) T. Inagaki, *J. Biochem.*, **32**, 57 (1940).

(10) C. Neuberg and I. S. Roberts, *Arch. Biochem.*, **20**, 185 (1949).

for terminal phosphorus on salt-protected DNA are less than 1 per 30 P.[11]

In the preliminary work just described it was observed that fibrous precipitates were obtained on addition of lead ion to the acid (*p*H 4) solutions of DNA. This precipitation was not observed prior to acidification of the solution. The present report is concerned with lead ion combination with DNA samples deliberately deformed or "denatured" by brief heating at *p*H 6 and moderate ionic strength. Lead–DNA interaction was studied in an effort to demonstrate the relative stabilization of the DNA by this ion; as the report demonstrates, lead ion can confer lability upon DNA as a consequence of its effective binding to one or more denatured forms of DNA.

Experimental

DNA from herring sperm (commercial) or from calf thymus[12] was used in the previous work.[11] This investigation used DNA from chicken erythrocyte prepared by a detergent procedure of Simmons,[13] which is abstracted below.

The chicken red cells are blended in a solution of sodium dodecyl sulfate (20%) and Versene (5 m*M*) in the Waring blendor. The resulting gel receives a little toluene for preservative. The *p*H is adjusted to 7.5 with ammonia. The gel is stored overnight at room temperature. Four volumes of a chilled solution containing Versene (10 m*M*), potassium chloride (0.2 *M*) and sodium xylene sulfonate (12%) are added. After brief blending the mixture is transferred to a beaker in an ice-bath. The mixture is adjusted to *p*H 4.3 with glacial acetic acid, with continuous stirring.

The protein–detergent residue is discarded after centrifugation (2000 RCF, 45 min., 0°). The *p*H is adjusted to 7.0 with ammonia. The clear supernatant solution is stirred during the addition of one volume of 90% isopropyl alcohol. The fibrous residue (nucleic acids) is recovered by centrifugation. This residue is dissolved in 0.05 *M* Versene, and an equal volume of sodium xylene sulfonate (80% w./v.) is added. The mixture is stirred an hour. Four volumes of Versene (0.01 *M*) are added. Stirring continues while the solution is chilled in an ice-bath; the *p*H is adjusted to 4.3, as above.

A series of vacuum filtrations through Celite 535, Hyflo Supercel, and Analytical filter aid are executed. The filtrate is cooled in ice with octanol present to reduce foam. Filtrations are terminated when the filtrate is "crystal clear." The nucleic acids are precipitated by the addition of one volume of isopropyl alcohol to the neutralized filtrate. The residue is dissolved in a mixture containing acetate buffer (0.3 *M*, *p*H 7) and Versene (0.01 *M*). The slow addition of isopropyl alcohol to a final concentration of 30% (v./v.) precipitates the ribonucleates. After centrifugation, the dropwise addition of isopropyl alcohol to 34% (v./v.) precipitates the DNA. The fractionation steps are repeated several times. The final DNA fibers are dried by rinsing with 95% ethanol and dry acetone. Acetone was removed *in vacuo* and the DNA was stored at −18°. In later procedures in our laboratory, we have substituted centrifugations (4×10^4 RCF, 4°, 60 min.) for the various filtration steps, since these were slow by our procedure and loss of yield was observed.

The preparation minimizes enzymatic degradation of DNA by the high level of detergent and Versene. The DNA does not contact acid or alkali except for the two protein-removal steps (*p*H 4.3, 0°). The standard DNA preparation used in this work has received minimal contact with acid (*p*H 4.3) since clarification proceeded by centrifugation rather than the long filtrations. This DNA was never subjected to low ionic strength.

Stock solutions of these preparations were stored at the 2.9 m*M* DNA P concentration level in 0.1 ionic strength. The ionic strength was provided by sodium chloride, since the lead ion concentrations studied were insufficient to precipitate lead chloride. The maximal lead ion concentration was 17 m*M*. The mixtures remained at approximately *p*H 6.

The DNA solutions (usually 1-ml. aliquots) were heated in a water-bath, under conditions such that about 15 seconds heating was necessary for arrival at temperature. Total heating time was either 60 or 120 seconds. The water lost by the sample tube was replaced. After the appropriate lead ion additions and a reaction time of 10 min., solutions were centrifuged, if necessary, at 600 × *g* for 10 min. Aliquots of the clear solution or the supernate were diluted 1:100 with 0.1 *M* NaCl and the absorbance at 260 mμ was determined.

Results

Table I presents the results of a study designed to show the completeness of reaction between lead ion (17 m*M*) and heated DNA (2.4 m*M* P). It is apparent that the heated and cooled DNA solutions show no precipitation and about 10% rise in absorbance. Heating and cooling, followed by the addition of lead ion results in complete precipitation of the DNA, if the heating temperature was 100°. If heating at 60° is used, no change occurs beyond a minor increase in absorbance of the DNA. If the same concentration of lead ion is established prior to the heating, complete removal of DNA is apparent after heating at 60°, with almost complete removal after treatment at 100°.

TABLE I

EFFECT OF LEAD ION ON HEATED DNA[a]

	Absorbance (260 mμ) of supernatant soln.		
	DNA alone	DNA + Pb + Ht.	DNA + Ht. + Pb
No heating	0.18	0.18	0.18
Heating, 60°, 2 min.	.19	.00	.19
Heating, 100°, 2 min.	.20	.02	.00
DNA (100°, 2 min.) plus stock DNA			.09[b] (.18)

[a] Chicken erythrocyte DNA, 2.9 μg.-atom P/ml. in 0.1 *M* NaCl. Heating: 2 minutes at 60 or 100°, as indicated. Samples were cooled in water-bath before Pb additions (last column). Lead additions: lead nitrate, to final concentration of 17 m*M*. Centrifugation in small centrifuge, 10 min. at 3000 r.p.m. Dilution of supernate: 1:100 in 0.1 *M* NaCl. Absorbance values are corrected for that of the lead nitrate, on the assumption that all of this salt remained soluble. No precipitation of lead chloride is observed. [b] One volume of stock DNA plus one volume of heated and cooled DNA plus two volumes of lead nitrate solution. Total volume was double that of other samples. Absorbance value corrected for total volume is given in parentheses.

The usual procedure consisted of combination of equal volumes of the DNA and the stock lead nitrate. The lowest numerical entry in the table shows the result of combination of two volumes of lead nitrate with single volumes of heated and unheated DNA. This mixture is comparable to any other test mixture, with the exception that total volume is doubled. The absorbance of this mixture was 0.09 before correction for total volume, or 0.18 after correction. The DNA samples were premixed before addition of lead ion, so that the result indicates that lead ion is able to precipitate heated DNA particles in the presence of unheated particles.

(11) E. L. Duggan, V. L. Stevens and B. W. Grunbaum, THIS JOURNAL, **79**, 4859 (1957).

(12) N. S. Simmons, S. Chavos and H. K. Orbach, *Federation Proc.*, **11**, 390 (1952); A. E. C. Report: U. C. L. A. 184 (1952).

(13) N. S. Simmons' method D., unpublished. Private communication. Dr. Simmons has given his kind permission to include this abstract of the procedure.

Viscosity changes following heating[14,15] or absorbance changes at various temperatures[16] indicate that temperatures up to 70° may be tolerated for many minutes. Temperatures above 80° are considered sufficient to produce a form of DNA which lacks its characteristic viscosity or absorbance values. The results of a series of treatments of the DNA at various temperatures are recorded in Table II. It may be noted that the characteristic dependence of DNA denaturation on temperature is found. The interesting feature of the results is that the formation of an insoluble lead salt is apparent after two min. heating.

Lead nitrate and lead formate may be used interchangeably in these experiments. Therefore, the precipitation of DNA in the presence of lead nitrate at 60° is attributed to the lead cation rather than to the nitrate anion. In addition, when DNA was heated in the presence of potassium nitrate (20 mM, 60°, 2 min.), no precipitate was obtained if the lead ion was added after cooling.

TABLE II

TEMPERATURE DEPENDENCE OF LEAD–DNA INTERACTION[a]

Temp., °C.	Heating time (sec.)	Results
79	60	Clear solution
85	60	Clear solution
87–89	60	Clear solution
85–87	120	Slight turbidity
91–93	120	Flocculent white ppt.

[a] DNA (2.9 μg.-atom P/ml.) in 0.1 M NaCl, heated as described, cooled. Lead nitrate was added to a final concentration of 17 mM.

Table III gives the results of an experiment designed to differentiate the lead–DNA precipitation from possible aggregation phenomena. If the DNA were incompletely dissolved or aggregated as a result of denaturation during preparation, such aggregates might sediment under these conditions in both the unheated and heated DNA samples. A number of conclusions may be drawn from consideration of the results shown in Table III. (a) The absorbance of unheated DNA is almost independent of the quantity of lead ion present. (b) The absorbance of heated DNA is close to that of unheated DNA in the absence of lead ion. This indicates that the thermal damage to the DNA molecule was not extensive, or else the absorbance changes at temperature were reversed on cooling. (c) The reaction is half complete at 4.2 mM and complete at 8.2 mM lead ion concentration. The reaction is reversible since the lead–DNA complex redissolves rapidly in 10 volumes 0.01 M disodium Versenate (pH 7).[17]

TABLE III

SEDIMENTATION OF Pb–DNA COMPLEX[a]

Lead nitrate $M \times 10^3$	Lead nitrate Pb/DNA-P	Absorbance of supernate at 260 mμ: Unheated DNA	Absorbance of supernate at 260 mμ: Heated DNA[b]
0	0	0.14	0.14
0.8	0.7	.14	.11
4.2	3.5	.16	.07
8.3	6.9	.14	.00
16.7	13.8	.13	.00

[a] Chicken erythrocyte DNA (stock concentration, 2.9 μg.-atom P/ml. in 0.1 M NaCl). [b] DNA heated to boiling for 1 min. at stock concentration and ionic strength and then cooled. Five-ml. aliquots of heated or unheated DNA were diluted to 12 ml. with 0.1 M NaCl and lead nitrate at the appropriate concentration. All samples were centrifuged in the Spinco model L preparative centrifuge, number 40 head, 40,000 r.p.m. (RCF_{av} 105,400), for 45 min. Aliquots of supernate were diluted 1:50 with 0.1 M NaCl for absorbance measurements. All absorbances are corrected for the appropriate controls.

A criticism has been made that the DNA samples employed have been subjected to degradation by acid (pH 4) and by drying with acetone. Other more recent preparations have shown similar precipitation with lead ion after deformation of the DNA. These preparations have never been dried nor treated with acetone. The pH 4 deproteinization step remains essential; its effect upon the DNA has been minimized by using acetate buffer, maintaining high ionic strength, and cooling during this step.

We are not yet prepared to assess the "nativity" or "intactness" of our preparations beyond the hypochromic, hypotiter criteria used in the previous paper.[11] "Native" and "intact" are vague terms, lacking consistent meaning among workers in the field. We consider that the lead ion test discriminates between the isolated, relatively "native" DNA and the DNA which has been deformed by brief treatments with heat, acid or alkali.

Discussion

It seems futile to present a detailed explanation of this phenomenon at this time. The findings represent a method of great significance in the comparison of DNA specimens prepared by a variety of methods. It may also be possible to purify DNA samples into the soluble and insoluble fractions by the technique which yielded the data of Table III. The mechanism of combination with briefly heated DNA to yield an insoluble product is unknown. It should be noted that the addition of lead ion before heating is responsible for the formation of the insoluble DNA complex at temperatures about 30° below those which are usually necessary to convert the DNA into a form which is precipitable with lead ion.

It is also significant that the various heating times are less than 2 minutes, in the period where no appreciable change in absorbance or viscosity has occurred. It seems reasonable to conclude that the hydrogen-bonded structure[18] has been distorted to a minimal extent by the heating procedure. It would be of great interest to obtain comparative physical and chemical data for lead-precipitated and control DNA samples. Without such data, we suspect that no appreciable change in viscosity or absorbance has occurred, once the lead ions have been removed completely.

It remains a possibility that similar procedures can discriminate between potentially labile DNA

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molecules and the bulk material. We have obtained preliminary evidence that denaturation by alkali or acid treatments for brief intervals deforms the DNA sufficiently for the formation of the lead complex. We have not yet investigated the precipitability of the products of enzymatic hydrolysis by DNase. Other cations may substitute for lead ion in the test. The effects of other cations or anions are under investigation.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF PENNSYLVANIA]

Synthesis of Bis-benzimidazoles

BY LILLIAN LI-YEN WANG AND MADELEINE M. JOULLIÉ[1]

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A number of new bis-benzimidazoles have been prepared. Substituents have been placed on both benzene rings of the benzimidazole nuclei. The chain linking the two benzimidazole units has been varied from simple alkane chains to substituted alkane chains or aryl chains (benzene ring). It was found that polyphosphoric acid is a useful medium for preparing bis-benzimidazoles. In difficult cases the use of a little concentrated sulfuric acid as a catalyst was beneficial.

The benzimidazole nucleus has been of considerable interest since it was noted that benzimidazole inhibits the growth of certain yeasts and bacteria.[2] The discovery of 5,6-dimethylbenzimidazole as a unit in vitamin B_{12} has increased this interest. A number of alkyl benzimidazoles have been tested and found to have some anti-vitamin B_{12} activity and some have been reported to have anti-viral activity also.[3] That structural modifications can produce marked effects on physiological activity has been shown by the test data on the substituted benzimidazoles and naphthoquinone imidazoles which have been synthesized in this Laboratory.[4] Some of these compounds exerted strong inhibitory action not only against purine and B_{12} requiring microörganisms but against folic acid-requiring organisms as well.

Structural modifications of the benzimidazole nucleus can be carried out in several ways. In the present investigation methods were developed to synthesize new bis-benzimidazoles where the two benzimidazole nuclei are united through their 2-positions either directly or through one or more atoms. Such systems can be modified not only by changing the nature and the number of the connecting atoms but by changing the nature of the substituents in the benzimidazole nuclei as well.

A review of the literature revealed the fact that a number of bis-benzimidazoles have been reported but apparently none of them have been screened for physiological activity. In the present study, greatest emphasis was placed on the preparation of substituted bis-benzimidazolylalkanes although a few other types were also prepared.

Phillips' method for the preparation of simple benzimidazoles is well known.[5] The same procedure can be used to prepare bis-benzimidazoles by refluxing two moles of diamine with one mole of a dibasic acid in 4 *N* hydrochloric acid. The method was modified by Shriner and Upson by using a much longer reflux period.[6] In the present work a further modification was made. It was found that the bis-benzimidazoles could be conveniently isolated as their dihydrochlorides by cooling the reaction solution after the refluxing period. The isolation of the dihydrochloride was preferable to neutralization of the whole solution because in general the dihydrochloride of the diamine is more soluble than the dihydrochloride of the bis compound. Thus a separation of most of the excess diamine can be accomplished in this step. The dihydrochloride was then treated with hot bicarbonate solution or aqueous ammonia to remove any benzimidazolylalkylcarboxylic acid which would normally be present.

This modification of Phillips' method gave fair yields in most cases. It failed, however, when used to condense 3-nitro-5-chloro-*o*-phenylenediamine with succinic, adipic and suberic acids. The polyphosphoric acid method, recently reported by Hein, Alheim and Leavitt for the preparation of simple benzimidazoles, was then tried.[7]

The polyphosphoric acid method not only worked for 3-nitro-5-chloro-*o*-phenylenediamine with succinic, adipic and suberic acids but was found to be convenient and fairly general giving about the same yields as the modified Phillips method. The polyphosphoric acid method failed in one case, with 5-nitro-*o*-phenylenediamine.

In the preparation of the bis-benzimidazolylethanes, although 3,4-diaminotoluene and 4-chloro-*o*-phenylenediamine were condensed with succinic acid to give fair yields of the bis compounds, it was found preferable to use succinic anhydride instead of the free acid for the other substituted diamines.

As the alkane chain between the two benzimidazole rings increased in length, the preparation of bis-benzimidazoles became more difficult. The use of polyphosphoric acid was the best method for the condensation of *o*-phenylenediamines with the longer chain dibasic acids. Substituted bis-benzimidazolyloctanes were not easy to obtain even by this method until it was noted that the addition of a small amount of concentrated sulfuric acid facilitated the reaction. Although this modification was not tried with all of the other dibasic acids, it would probably be helpful in those cases also.

(1) To whom all inquiries should be addressed.

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