merized DNA, since internal configuration seems to influence DNase affinity as well as methyl green affinity. It is suggested that DNase acts only upon the point of cleavage of the DNA molecule, causing loss of affinity for methyl green at this point only. The configuration of the rest of the molecule, unlike the effect of heat and X-ray, is thought to be unaffected until depolymerization has progressed to small fragments.

Effect of Methyl Green Binding on Viscosity of DNA Solution.—Methyl green was found to slightly increase viscosity (Table I) without significantly altering sedimentation velocity. This suggests that the methyl green radical, which attaches to the DNA molecule at two sites,[4,9,13] may produce some linking between adjacent DNA molecules. This type of change in structure of DNA, however, does not appear to alter susceptibility to enzymatic depolymerization.

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NEW ORLEANS, LA.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF ARKANSAS]

On the Soluble Nucleotides of Liver and Muscle[1,2]

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Ion-exchange chromatography of the barium-insoluble fraction of the acid-soluble nucleotides of liver has shown the presence of a mononucleotide which appears to be a pyrimidine derivative different from cytidylic or uridylic acid. Its phosphate group is liberated by enzymes which are specific for monophosphates and 5'-derivatives. This nucleotide has been found in both rat and rabbit liver, but is absent from rabbit muscle. Evidence has been obtained by ion exchange chromatography for the presence in liver and muscle of di- and polyphosphate nucleotide derivatives different from ADP and ATP.

The work to be reported here is a study that was made as preliminary to an investigation of the time-course of the distribution of tracer phosphate between the individual phosphate groups of ADP and ATP in liver and muscle. The procedure used initially for the separation and isolation of these two compounds was that described by Cohn and Carter[3] for the separation of the components of commercial preparations of ATP. This consists of the adsorption of the nucleotide derivatives from ammoniacal solution onto a column of Dowex-1 anion exchange resin, the height of which is equal to the diameter, followed by elution successively with 0.003 *N* HCl, 0.01 *N* HCl plus 0.02 *M* NaCl, and 0.01 *N* HCl plus 0.2 *M* NaCl. These solutions elute AMP, ADP and ATP, respectively.

Columns of these dimensions failed to effect the desired separation of the soluble nucleotide fractions of trichloroacetic acid extracts of tissues. Increasing the ratio of height to diameter of the column of resin did lead to the desired separations, and also revealed the presence of other nucleotides and their polyphosphate derivatives. Most of the experiments have been carried out with columns of 250–400 mesh Dowex-1 resin, 18 cm. high by 1.1 cm. in diameter. Three additional fractions have been obtained from both liver and muscle extracts; these appear to be mixtures of nucleotide di- and polyphosphates. The composition of these fractions is still under investigation.

Liver extracts contain, in addition to these fractions, a substance which shows the properties of a pyrimidine nucleotide, but does not correspond in elution characteristics or absorption spectrum to either cytidylic or uridylic acid. These four nucleotide fractions from liver account for about 20% of the total specific absorption at 260 mμ shown by the crude extracts; the three fractions from muscle account for about 10% of such absorption.

These studies have been carried out on rabbit muscle and liver, and on rat liver. Qualitatively similar findings were obtained from the livers of both species.

Experimental

The rats were anesthetized with pentobarbital, the livers excised and dropped into a mixture of Dry Ice and ether. The rabbits were anesthetized with pentobarbital supplemented with ether. The thigh muscles were dissected as free as possible without cutting any major blood vessels, excised rapidly and immediately dropped into the freezing mixture. The liver was then excised and frozen in the same way. All subsequent operations except the determinations of optical density of the solutions were carried out in a cold room maintained at 1–2°. The tissues were broken up into small pieces in a tissue crusher chilled with Dry Ice, and the pieces then placed in a Waring blendor with 5 volumes of 10% trichloroacetic acid. The blendor was run until a homogeneous suspension was obtained; this required 2 to 3 minutes with liver and 5 minutes with muscle. The suspension was centrifuged immediately, and the residue washed once with 3 volumes, and once with 2 volumes of 5% trichloroacetic acid. Glycogen was precipitated from the combined supernatants by the addition of somewhat more than an equal volume of 95% ethanol. This precipitate was removed by centrifugation half an hour after the addition of the ethanol. The fraction of barium-insoluble compounds was separated from the clear supernatant by the use of barium hydroxide and calcium acetate, as described previously.[4] It was found that the fraction of alkaline-earth soluble compounds, obtained by the addition of 4 volumes of ethanol to the combined supernatants from the precipitations with barium and calcium, contained appreciable quantities of material showing absorption at 260 mμ. This material was dissolved in a small volume of 0.2 *N* HCl, the solution made just alkaline to phenolphthalein by cautious addition of powdered barium hydroxide, calcium acetate solution added, and the mixture allowed to remain in

(1) Work performed under contract (AT-40-1) 1521, between the U. S. Atomic Energy Commission and the University of Arkansas.

(2) These experiments were initiated while J. S. and L. L. were at Brookhaven National Laboratory, Upton, N. Y.

(3) W. E. Cohn and C. E. Carter, THIS JOURNAL, **72**, 4273 (1950).

(4) J. Sacks, *J. Biol. Chem.*, **181**, 655 (1949).

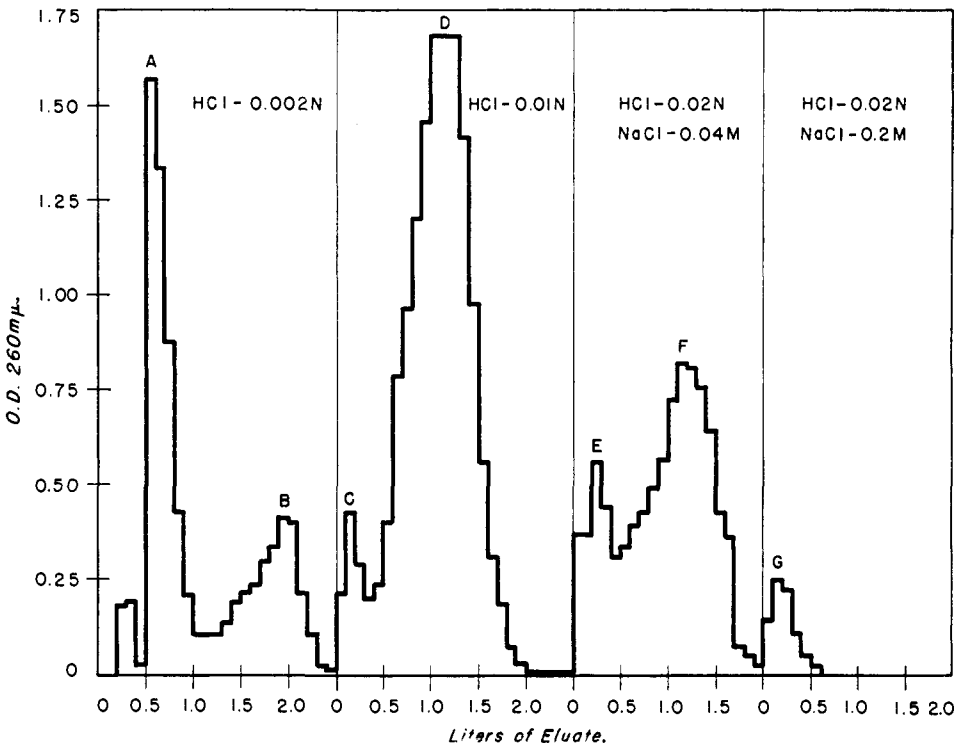

Fig. 1.—Elution pattern of nucleotides from barium-insoluble fraction of TCA extract of rabbit liver. Ordinates are optical density readings of successive 100-ml. fractions of eluate of material adsorbed on column of Dowex-1 18 cm. high × 1.1 cm. in diameter. The first unlabeled peak is not constant in appearance. The other peaks represent, respectively: A, adenosine-5′-phosphate; B, the new pyrimidine nucleotide; C, a nucleotide diphosphate fraction containing ADP and some compound of unknown composition; D, ADP; E, a mixture of nucleotide polyphosphates; F, ATP; and G, a mixture of polyphosphate derivatives.

the cold room overnight. The precipitate which appeared was separated by centrifugation, washed once with water, and added to barium and calcium salts obtained in the initial fractionation.

The combined alkaline earth precipitates were dissolved in the minimum volume of 0.2 *N* HCl, and the barium and calcium removed by passage through a column of Dowex-50 cation exchange resin (sodium form) 6 cm. high by 2 cm. in diameter. The resulting solution was brought to *p*H 8 to 9 by the addition of dilute ammonia, diluted to a suitable volume such that the chloride concentration did not exceed 0.01 *M*,[5] the optical density at 260 mμ determined, and the phosphate compounds adsorbed on columns of Dowex-1 resin, 250–400 mesh (chloride form) 18 cm. high by 1.1 cm. in diameter. The columns were washed with water and the orthophosphate eluted with 1 liter of the mixture of 0.025 *M* NH_4Cl and 0.01 *M* $K_2B_4O_7$ used by Khym and Cohn[6] for the separation of the phosphorylated sugars. The function of borate in the elution of orthophosphate is not clear, but this mixture gave clearly defined elution patterns with sharp peaks, whereas 0.025 *M* NH_4Cl itself was not an effective eluant. The nucleotides were then eluted with the following eluants[5] used successively: 0.002 *N* HCl, 0.01 *N* HCl; 0.02 *N* HCl plus 0.04 *M* NaCl, and 0.02 *N* HCl plus 0.2 *M* NaCl. The elution rate was approximately 50 ml. per hour, and two-hour collections were made with an automatic fraction collector. The progress of the elution was followed by measurements of the optical density at 260 and 280 mμ in a Beckman model DU spectrophotometer. Each fraction was concentrated by making the eluate ammoniacal, readsorbing on a column of Dowex-1 resin 2 cm. high by 1.1 cm. in diameter, and eluting with a solution of lower *p*H and higher ionic strength than that which had served for the initial elution.

Results

Figure 1 is a representative elution pattern obtained with material from the liver of a rabbit. Two clearly defined peaks of optical density were obtained with each eluant except the one of highest ionic strength. The elution pattern obtained with rat liver extracts was qualitatively the same as that obtained with rabbit liver extracts. Extracts of rabbit muscle gave patterns which differed qualitatively in that only one peak was obtained with 0.002 *N* HCl.

The middle fractions of peaks A, D and F, concentrated by adsorption on short columns of Dowex-1 and elution with small volumes of eluants of higher ionic strength and lower *p*H, were identified as adenosine-5′-phosphate, ADP and ATP, respectively, by the following procedures: comparison of the elution patterns with those of known materials, determination of the ultraviolet absorption spectra, determination of the adenine:ribose:phosphorus ratios, and of the ratio of acid-labile to acid-stable P of the ADP and ATP.

Peaks C, E and G are mixtures, as shown by the progressive fall in the ratio of optical densities of 280 and 260 mμ of successive 100-ml. portions of the eluate, from about 0.6 to 0.2. These fractions all contain some phosphate groups readily hydrolyzable by acid (1 *N* H_2SO_4 at 100° for 15 minutes) as well as acid-stable phosphate. Peaks C and E probably contain some ADP and ATP, respectively, since the elution curve for a single substance is symmetrical. Further studies are in progress in the attempt to separate the components of these mixtures.

(5) W. E. Cohn, *J. Cell. Comp. Physiol.*, **38**, Suppl. 1, p. 21 (1951).
(6) J. X. Khym and W. E. Cohn, THIS JOURNAL, **75**, 1153 (1953).

The material represented by peak B appears to be a single substance. The ratio of optical density at 280 mμ to that at 260 mμ is constant at 0.42 for all the 100-ml. fractions of eluate. Readsorption of the material of peaks A and B on a column of Dowex-1 resin in the formate form and elution with 0.1 *N* formic acid, yields two distinct peaks, as shown in Fig. 2. Under these circumstances which are essentially those used by Cohn and Volkin[7] for the separation of the isomeric mononucleotides, the new compound is eluted before adenylic acid. The absorption maximum of the new substance at *p*H 2 is 262–263 mμ, and the minimum is at 235 mμ. The molar extinction coefficient is approximately 10,000 at the point of maximum absorption, on the assumption that the substance is a mononucleotide. The absorption spectrum at *p*H 7 in phosphate buffer is practically identical with that at *p*H 2. At *p*H 12 the point of minimum absorption is shifted to 245 mμ, and the molar extinction coefficient at 263 mμ is reduced by about 40% below the value at *p*H 2. The ratio of optical density at 250 mμ to that at 260 mμ is 0.72 at *p*H 2, and 0.82 at *p*H 12. The ratio at 280 to 260 mμ at *p*H 12 is 0.28.

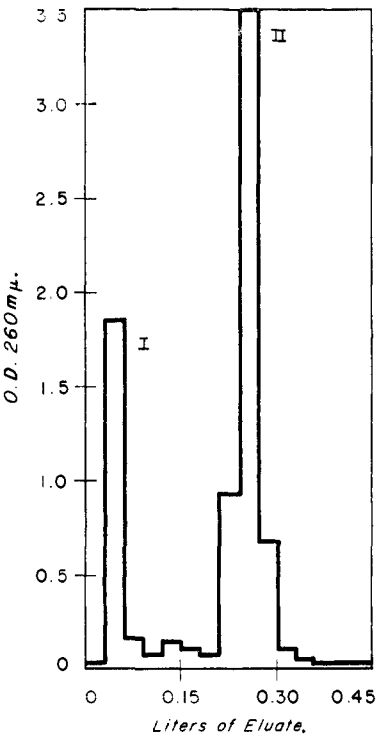

Fig. 2.—Elution of mixtures of peaks A and B of Fig. 1 from a Dowex-1 column, formate form, by 0.1 *N* formic acid; elution rate 10 ml. per hour. Peak I is the new pyrimidine nucleotide; peak II is adenosine-5′-phosphate.

The new substance is hydrolyzed extremely slowly by 1 *N* sulfuric acid at 100°, about 3% of the phosphate being liberated per hour under these conditions. The pentose equivalent found by the orcinol reaction is 0.15 to 0.2 per equivalent of P. Enzymatic studies with prostatic phosphatase and with a specific 5′-nucleotidase show that the phosphate is all present as monoester of a 5′-nucleotide.

The absorption spectrum of the new substance is quite different from that of cytidylic acid, but reasonably close to that of uridylic acid. However, when this substance was mixed with uridine-2′-phosphate, the mixture adsorbed on a Dowex-1 column in the chloride form, and elution carried out with dilute HCl, two separate peaks were obtained. The new substance was eluted with 0.002 *N* HCl, 2 liters of which failed to elute the uridylic acid; the latter substance was then eluted with 0.003 *N* HCl, with the peak at 700 ml. These data are in agreement with those of Cohn,[5] which indicate also that HCl does not separate the various uridylic acids from each other.

The material represented by peak G showed ratios of optical density at 280 mμ to that at 260 mμ decreasing from 0.5 to 0.3 in successive 100-ml. portions of the eluate. The ratio of acid-labile to acid-stable P was not constant; material obtained from different liver and muscle samples gave values ranging from 2.5 to 4.2. The orcinol reaction indicated the presence of approximately one equivalent of pentose per equivalent of acid-stable P.

Discussion

The absorption spectrum of the new mononucleotide is sufficiently close to that of uridylic acid to suggest that the substance may be a derivative of uracil. The report by Hirlbert[8] of the isolation of uridine-5′-phosphate from rat liver, and the finding by specific enzymatic hydrolysis that the new substance reported here is a 5′-derivative, point in the same direction. However, the elution characteristics from Dowex-1 resin are different from those of the known uridylic acids. The new substance precedes uridylic acid in elution by HCl, and precedes adenylic acid in elution by formic acid. Cohn and Volkin[7] found that the adenylic acids preceded the uridylic acids in elution from a formate column. The identity or lack of identity with uridylic acid can be established only when sufficient material is available for isolation and characterization of the base.

The nature of the di- and polyphosphate derivatives found in these studies must await isolation of pure substances. The uridine diphosphoglucose isolated by Caputto, *et al.*,[9] and the uridine diphosphate which Hirlbert[8] has found in liver may well be present in some of the mixed fractions reported here.

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(7) W. E. Cohn and E. Volkin, *Nature*, **167**, 483 (1951).

(8) R. B. Hirlbert, *Federation Proc.*, **12**, 222 (1953).

(9) R. Caputto, L. F. Leloir, C. E. Cardini and A. C. Paladini, *J. Biol. Chem.*, **184**, 333 (1950).