Dephosphorylation of Nucleic Acids, Mononucleotides and Casein by Lanthanum and Cerium Hydroxide Gels

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E. Bamann[1] has found that certain metal hydroxide gels, especially those of lanthanum and cerium, have catalytic effect on the hydrolysis of phosphate esters. Since nucleic acids are also phosphate esters, we studied the behavior of yeast ribonucleic acid towards lanthanum hydroxide gel and have found that it is easily dephosphorylated.[2] Independently of us, and probably of Bamann, K. Dimroth and his coworkers quite recently reported the similar effects of metal hydroxide gels.[3] J. E. Backer and F. W. Allen,[4] on the other hand, showed that pyrimidine nucleotides could be hydrolysed into nucleosides and inorganic phosphate under similar conditions.

The present paper deals with our recent studies on the catalytic effects of lanthanum and cerium hydroxide gels.

Materials

Two preparations, free acid and sodium salt, of brewer's yeast RNA, designated as RNA Preparation 1 and 2, were used. These were extracted by the method of Clarke and Schryver.[5] In obtaining Preparation 2, the chloroform method of Sevag et al.[6] was used. The phosphorus content of the two preparations was 6.5 and 8.7 per cent, respectively. The highly polymerized preparation of the sodium salt of calf spleen DNA, containing 7.4 per cent phosphorus, was

(1) E. Bamann, *Angew. Chem.*, **52**, 186 (1939).
(2) F. Egami and M. Shimomura, *Science (Japan)*, **18**, 472 (1948).
(3) K. Dimroth, L. Jaenicke and D. Heinzel, *Ann. Chem.*, **566**, 206 (1950). K. Dimroth, L. Jaenicke and I. Vollbrechtshausen, *Z. physiol. Chem.*, **289**, 71 (1952).
(4) J. E. Backer and F. W. Allen, *J. Biol. Chem.*, **188**, 59 (1951).
(5) G. Clarke and S. B. Schryver, *Biochem. J.*, **11**, 319 (1937).
(6) M. G. Sevag, D. B. Lackman and J. Smolens, *J. Biol. Chem.*, **124**, 425 (1938).

kindly supplied by Dr. A. Sibatani. Desoxyribo-, a-ribo-, b-ribo and 5'-ribocytidylic acid were kindly supplied by Dr. W. E. Cohn. The phosphorus content of commercial casein prepared by the method of Hammersten was 0.80 per cent.

Lanthanum carbonate was prepared from industrial lanthanum oxide. Since the latter contained silicates and metaphosphates, which hinder the action of lanthanum hydroxide and disturb the estimation of phosphoric acid detached from substrates, these impurities were removed during the course of preparation of the carbonate by heating with hydrochloric acid and then with sodium hydroxide and finally by treating the mixture with ammonium molybdate. Cerium carbonate was likewise prepared from industrial cerium oxide.

Experimental Conditions

The reaction mixture in a typical experiment for the measurement of dephosphorylation of RNA: 100 mg. of lanthanum carbonate were suspended in distilled water and dissolved by the addition of hydrochloric acid in a 50 cc. Erlenmeyer flask. Then it was adjusted to *p*H 8.0 by aqueous ammonia, thus lanthanum hydroxide was precipitated. To this suspension, 1 cc. of 1 per cent RNA solution, 10 cc. of 0.1 N sodium veronal-hydrochloric acid buffer, *p*H 8.0, and water were added till the final volume was 20 cc. RNA formed additional precipitate with lanthanum ion, known as a powerful precipitating reagent for nucleic acids.[7] The flask was rubber-stoppered, shaken vigorously to make the suspension homogeneous and immersed in a water-bath, which was held at desired temperature (time zero.). The mixture was shaken occasionally during the hydrolysis to avoid precipitation. Aliquots of 1 to 2 cc. were withdrawn at various time intervals, cooled to room temperature if necessary, and analysed for inorganic phosphate. Similar conditions were also used when the dephosphorylation of phosphoric esters other than RNA were tested.

Analytical Methods

Total Phosphorus.—The phosporus content of the various preparations was measured by the method of Lieb and Wintersteiner.[8]

Inorganic Phosphate.—In the reaction mixtures, phosphate was found only in the precipitate and was completely absent in the supernatant. Samples for analysis, therefore, were withdrawn carefully avoiding uneven sampling.

In reaction mixtures containing nucleic acid or protein, the bulk of these substances was precipitated by the addition of an equal volume of 10 per cent trichloroacetic acid. This technique permitted the direct colorimetric determination of the phosphate in the supernatant according to the method of Fiske and Subbarow.[9] The decrease of the colour intensity due to the presence of lanthanum was negligible within concentrations used. A slight turbidity which appeared on addition of the molybdate was removed by centrifugation.

Reducing Power.—If the glycosidic linkage in nucleosides is broken during the hydrolysis of RNA, the appearance of a reducing group is to be expected. This was measured by the ferricyanate reduction method of Fujita and Iwatake,[10] in which the lanthanum contained in the test samples was removed together with cadmium hydroxide.

Orcinol Reaction for the Estimation of RNA and Diphenylamine Reaction for the Estimation of DNA—Methods described by Kerr and Seraidarian[11] and by Dische[12] were used respectively; the presence of lanthanum did not disturb the estimations.

Experimental

Effect of Temperature.—Fig. 1 shows the dephosphorylation reaction of RNA by lanthanum hydroxide gel at various temperatures. It was observed that 95 per cent of the total phosphate groups of RNA were transformed into inorganic phosphate in 24 hours both at 90° and at 100°. In a control experiment at 80°, in which lan-

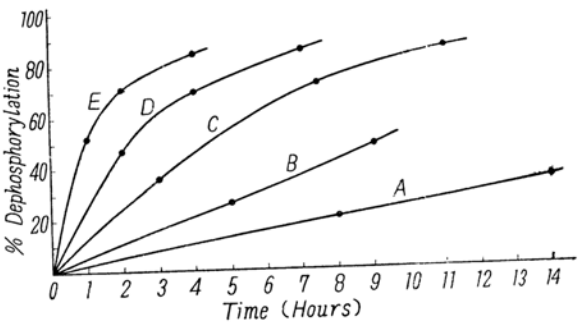

Fig. 1.—Effect of temperature on the hydrolysis of ribonucleic acid under the influence of lanthanum hydroxide. Each reaction mixture contained 10 mg. of RNA Preparation 2, lanthanum hydroxide (from 100 mg. of lanthanum carbonate), and 10 cc. of 0.1 N veronal buffer, *p*H 8.0, the total volume 20 cc. Curves A, B, C, D and E are at 60°, 70°, 80°, 90° and 100°, respectively. Curves D and E reached at 95 per cent dephosphorylation in 24 hours.

(7) E. Hammersten and T. Theorell, *Acta Med. Scand.*, **68**, 226 (1928).

(8) H. Lieb and O. Wintersteiner, *Mikrochemie*, **2**, 78 (1924).

(9) C. H. Fiske and Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

(10) A. Fujita and D. Iwatake, *Biochem. Z.*, **242**, 43 (1932).

(11) S. E. Kerr and K. Seraidarian, *J. Biol. Chem.*, **159**, 211 (1945).

(12) Z. Dische, *Mikrochemie*, **2**, 26 (1930).

thanum was omitted, no formation of inorganic phosphate was observed. On the other hand, the dephosphorylation of RNA was very slow at low temperatures. The amount of inorganic phosphate was only 2 per cent at 28° and 0 per cent at 5° after 5 days.[13]

The activation energy of the dephosphorylation reaction of RNA under the influence of lanthanum hydroxide was calculated from Fig. 1. The reaction was approximately of the first order, at least before 80 per cent dephosphorylation. Log k and $1/T$ were in linear relation between 60° and 100°. The activation energy was 20,200~20,400 cal.

Effect of pH. — The influence of pH on the lanthanum hydroxide is illustrated in Fig. 2. Optimal pH lies between 8.0 and 9.0.

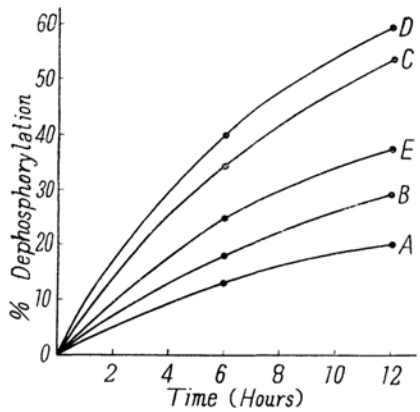

Fig. 2.—Effect of pH on the hydrolysis of ribonucleic acid under the influence of lanthanum hydroxide. Curves *A*, *B*, *C*, *D* and *E* are at pH 6.0 (citrate buffer), 7.0 and 8.0 (veronal buffer), 9.0 and 10.0 (ammonium buffer), respectively. Temperature 70°. Other conditions similar to Fig. 1.

Effect of the Quantity of Lanthanum hydroxide.—The catalytic effect of lanthanum hydroxide increased with increasing quantity (Fig. 3). The atomic ratio of lanthanum/phosphorus in the reaction mixture in which 10 mg. of lanthanum carbonate was used was approximately 1 (Curve *B*). After 40 hours Curves *B*, *C* and *D* reached 100 per cent, whereas Curve A barely reached 70 per cent. This was understandable since the phosphate formed during the hydrolysis of RNA hindered the catalytic effect by firmly attaching itself to lanthanum.

Natures of the Hydrolysis Product—RNA was almost completely dephosphorylated under the relatively mild conditions, and the nucleosides, the dephosphorylation product, were expected to remain unaltered under these conditions. The

(13) The data at low temperatures reported in the previous paper (2) had been slightly higher, since the inorganic phosphate had been estimated by the method of Bell and Doisy, and hence the colour due to silicates had been contaminated.

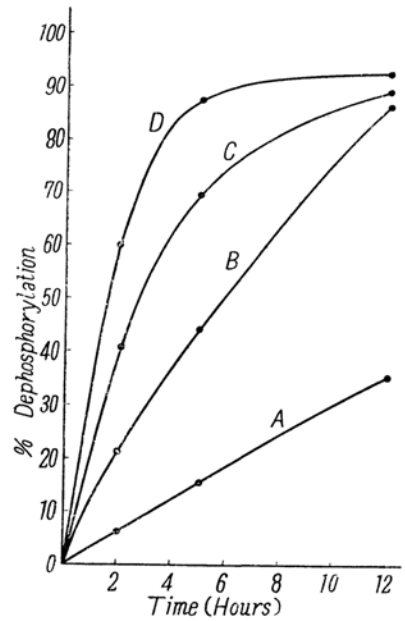

Fig. 3.—Effect of the amount of lanthanum hydroxide on the hydrolysis of ribonucleic acid. Curves *A*, *B*, *C* and *D* were obtained with lanthanum hydroxide produced respectively from 5, 10, 30 and 100 mg. of lanthanum carbonate. Temperature 90°. Other conditions similar to Fig. 1.

Table 1

Reducing Power and Colour Intensity of Orcinol Test during Dephosphorylation of RNA

Time, hrs	Dephosphorylation, %	Reducing Power† mg. glucose	Orcinol Test†† %
0		0.00	103
1		0.00	100
12		0.00	100
25		0.00	100
39		0.00	97
43	98	0.01	97
Calculation		1.25*	

The reaction mixture contained 50 mg. of RNA Preparation 2, lanthanum hydroxide obtained from 500 mg. of the carbonate, and 25 cc. of 0.1 M veronal buffer, pH 8.0, in a total volume of 50 cc. Temperature, 80°. 3 cc. of reaction mixture was used for each estimation of reducing power.

† Reducing power measured with 3 cc. aliquots and expressed as mg. glucose.

†† The colour intensities measured were compared with the standard RNA solution containing RNA in the same concentration as the original reaction mixture.

* On the complete hydrolysis of the glycosidic linkages.

reducing power of ribose should appear, if the glycosidic linkages in the nucleosides were broken; Table 1 shows that no reducing group was liberated. The colour intensity of the orcinol test for ribose also remained constant within experimental error.

Dephosphorylation Process and Confirmation of Ribonucleosides as the Final Products by Means of Periodate Oxidation.—The splitting of linkages between the bases and ribose groups may be detected by the periodate test.[14] One mole of periodate should be consumed per one mole of nucleosides, if these linkages remain intact, whereas, 4 moles should be consumed, if the breakdown is complete. Moreover, the course of the hydrolysis of RNA might be investigated by this periodate oxidation method, since 5'-ribonucleotides consume one mole of periodate per mole, but 2'- and 3'-ribonucleotides would not. For example, the formation of periodate consuming products would precede the dephosphorylation when RNA is hydrolysed through the intermediate 5'-ribonucleotides.

Since measurement was greatly disturbed by the existence of lanthanum salt, samples were mixed with an equal volume of 0.2 M phosphate buffer, *p*H 7.0, thus inducing the complete precipitation of lanthanum in the form of insoluble phosphate. After standing for about 10 minutes and centrifugation, aliquot of these supernatants was used for the periodate test. Another aliquot of these supernatants was examined by the orcinol test for ribose. The result of this test showed that the amount of RNA removed together with lanthanum was relatively small (Column 3, Table 2). Table 2 shows that the consumption of periodate precedes the dephosphorylation. It may be explained by the fact shown below (see Table 3) that the phosphate at 2'- or 3'-carbon atom is split off preferentially in comparison to that at 5'-carbon atoms. The fact that the final product consumes approximately one mole of periodate per mole of total phosphorus confirms the already mentioned finding that nucleosides are the final hydrolysis product.

Preparation of Ribonucleosides.—60g. of lanthanum carbonate were suspended in distilled water and dissolved by the addition of distilled hydrochloric acid. After precipitating lanthanum hydroxide from the solution by the addition of concentrated aqueous ammonia, the precipitate was washed thoroughly with dilute aqueous ammonia by centrifugation in order to remove the chlorine ion. The precipitate was finally suspended in distilled water. To this lanthanum hydroxide suspension was added RNA solution containing 20 g. of RNA Preparation 1. This mixture was adjusted at *p*H 8 to 9 with aqueous ammonia or with sulphuric acid. Total volume was about 1.5 liter. The mixture was heated to 80° and maintained at this temperature for about 40 hours. Aqueous ammonia was added occasionally to prevent lowering of *p*H. Then, the precipitate was filtered off and washed with 1.5 liter of hot water. (Protein, contained in the RNA Preparation, was adsorbed to, and inorganic phosphate, produced by the hydrolysis of RNA, was also bound with lanthanum hydroxide. Nucleosides went into the filtrate.) Lanthanum in the filtrate was precipitated quantitatively by the addition of oxalic acid and filtered by suction. The filtrate was concentrated to a volume of 300 cc. under reduced pressure at about 50° and then filtered while warm. Guanosine settled out from the filtrate in long needles upon freezing overnight. This was recrystallized from hot water. The yield was 2.1 g. Picric acid solution was then added to the filtrate of guanosine until there was no further precipitation of adenosine picrate. The picrate was filtered off and a second crop was obtained when the filtrate was concentrated to 400 cc. and allowed to stand in a refrigerator. The picrate was crystallized from hot water in the form of platelets. The yield was 4.0 g. Finnlly cytidine picrate was obtained in the form of long crystalline needles from the filtrate of adenosine picrate by the method of Levene.[15] The yield was 3.2 g.

Behaviour of Desoxyribonucleic Acid.—No dephosphorylaton of DNA was observed under

Table 2

Periodate Consumption by Hydrolysis Product in the Course of Dephosphorylation of RNA

Time, hrs	Dephosphorylation, %	Colour intensity of orcinol test, %	Periodate, consumption mole	Periodate consumption Colour intensity of orcinol test, mole
0	0	73	0.025	0.034
2	25	78	0.59	0.76
5	54	96	0.84	0.87
8	72	96	1.06	1.10
23	99	104	1.22	1.17

The reaction mixture contained 100 g. of RNA Preparation 2 and lanthanum hydroxide obtained from 300 g. of the carbonate. Total volume, 20 cc.; *p*H, 8.0; temperature, 80°. No buffer was used and occasional addition of N sodium hydroxide prevented the lowering of *p*H.

(14) R. Adams, "Organic Reaction," New York, **2**, 341 (1944).

(15) P. A. Levene, *J. Biol. Chem.*, **25**, 103 (1916).

the same conditions as shown in Curve 3 in Fig. 1 even after 48 hours. The supernatant of the reaction mixture at 48 hours gave negative diphenylamine test, although, in the case of RNA, the colour intensity of the orcinol test of the supernatant increased with increasing dephosphorylation of RNA. DNA, even after the depolymerization by heating in 0.25 N sodium hydroxide at 80° for 20 minutes, was not dephosphorylated under the influence of lanthanum hydroxide.

Dephosphorylation of Nucleotides.—Contrary to DNA, desoxyribocytidylic acid was dephosphorylated. Table 3 shows the dephosphorylation of desoxyribo- and ribonucleotides. b-Cytidylic acid is dephosphorylated more easily than a-cytidylic acid and 5'-cytidylic acid most slowly.

Table 3

Dephosphorylation of Various Cytidylic Acids

Time, hrs.	Desoxyribo-, %	a-Ribo-, %	b-Ribo-, %	5'-Ribo-, %
3	60	57	89	30
6	88	85	97	43
Control				
12	0	3.0		

The reaction mixture contained lanthanum hydroxide obtained from 30 mg. of the carbonate, nucleotide (1.92 mg. desoxyribo-; 2.08 mg. a-ribo-; 2.31mg. b-ribo-; 2.05mg. 5'-ribocytidylic acid, in each case), and 1 cc. of M ammonium buffer, *p*H 9, and the volume was made to 12 cc. with distilled water.

Table 4

Catalytic Effect of Lanthanum Hydroxide on Dephosphorylation of Casein

Time, hrs	Dephosphorylation at 25°, %	Dephosphorylation at 70°, %	Control, at 70°, %
18.5		71	20
21.5	0		
42.5		98	34

Each reaction mixture contained 50 mg. of casein, lanthanum hydroxide obtained from 150 mg. of the carbonate, 1 cc. of M ammonium buffer, *p*H 9, and the total volume was made to 10 cc. with water. In the control experiment, water replaced lanthanum hydroxide.

Dephosphorylation of Casein.—Casein was not dephosphorylated at room temperature, 25°, as Bamann has shown (1). But at 70°, the catalytic effect of lanthanum hydroxide was observed (Table 4).

Summary

1. The dephosphorylation reaction of RNA by lanthanum hydroxide gel was further studied. The activation energy of the reaction is 20,200~20,400 cal. Optimal *p*H is between 8 and 9.

2. RNA is hydrolysed almost completely into nucleosides and inorganic phosphate at 90° in 40 hours at *p*H 8. The formation of nucleosides as the final product of hydrolysis was shown from the reducing power measurements and periodate consumption of products.

3. DNA is not dephosphorylated at all under conditions where RNA is almost completely dephosphorylated. This may be related to the structural difference of DNA and RNA.

4. Preparative method for ribonucleosides from RNA under catalytic effect of lanthanum hydroxide was described. Ribonucleosides are obtained in good yields.

5. Desoxyribo-, a-ribo-, b-ribo-, and 5'-ribocytidylic acid are all subject to catalytic dephosphorylation. 5'-Ribocytidylic acid is dephosphorylated most slowly.

6. Cerium hydroxide can be used as catalyst of the reaction in the same way with lanthanum hydroxide.

7. The dephosphorylation of casein is considerably accelerated by lanthanum hydroxide at 70° but not at 25°.

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