through ribose-1-phosphate, hydrolysis should be an exact index of isomerization.

Although the conditions used to obtain ribose phosphate from guanylic acid *via* xanthylic acid were relatively mild, the absence of any manipulations to effect a separation of the nucleotide isomers plus the fact that hydrolysis is always accompanied by isomerization (see Table I and Fig. 2), makes it probable that at least two ribose phosphates were produced. Further isomerization of these is possible during the prolonged incubation, and there is no evidence of any attempt to recrystallize or otherwise separate the product isomers. Thus it is likely that both were present. In the case of adenylic acid, it is probable that the *b* isomer was indeed used as the starting material. However, the more drastic methods used to degrade it (attested to by the inorganic phosphate found) make it probable that isomerization took place to yield more than one ribose phosphate isomer. If these preparations (or that from guanylic acid) isomerized at the ribose phosphate stage, ribose-2-, -3- and -4-phosphates would all be present and the reduction of these to ribitol phosphate, even if no further isomerization took place, would yield a combination of optical inactivity (the -3-) and a racemate (the -2- and -4-) as pointed out by Brown and Todd.[11] In this connection, it should be noted (see, for example, Fig. 2) that it requires only about 100 minutes at 100° for ribose-3-phosphate, at its own *p*H (*i.e.*, with Dowex-50 present), to produce equal amounts of 2- and 4-isomers, each about half of the ribose-3-phosphate remaining. This fact and the preponderance of the 3-compound which we observe regardless of starting product (if the conditions are such as to degrade the bulk of the starting nucleotide) indicate that the optical inactivity of the Levene and Harris ribitol phosphate was probably due either to the production of a racemate[11] or to the unwitting selection of ribose-3-phosphate from the mixture and not to the unintentional preservation of isomeric integrity, even if initially present, throughout the degradation to yield ribitol-3-phosphate.[2a]

The existence of inorganic phosphate in preparations of ribose phosphate by nucleotide hydrolysis can be ascribed to acid attack on the phosphate linkage only if alkaline degradation of the product during workup has been avoided. Experiments reported elsewhere[31] indicate a greater degree of alkali lability of ribose-3-phosphate (and the 5-isomer) than of free ribose itself, with the -2-phosphate being more stable than ribose. Alkali degradation releases inorganic phosphate and may thus have been the source of this degradation product in some of the older precipitations of ribose phosphate(s), using barium at high *p*H.[2a,b]

Since similar results have been obtained with guanylic acid (that is, more ribose-2-phosphate from the hydrolysis of guanylic acid *a*, more of the -3- from the *b*), it would appear that both *a* purine nucleotides are the -2′-phosphates of the respective nucleosides while the *b*'s are the -3′-phosphates. The same order of ion-exchange elution (5′, 2′, 3′)[10] is observed in the pyrimidine nucleotides,[19,42] which would indicate the dependence of this order upon the *pK*'s of the ionizable groups.[10,16b,25] It is already known that the 5′-phosphoinosine precedes the 2′- and 3′-isomers[10a]; hence, it may be inferred that the inosinic acids *a* and *b* derived from commercial yeast adenylic acid are also 2′- and 3′-phosphoinosines, respectively. The similarity in physical properties of the corresponding adenylic and cytidylic isomers[25] already indicated and the derivation of the uridylic acid isomers from the cytidylic acid isomers,[18] together with the similarities in enzymic susceptibility of all *a*'s and all *b*'s,[19a] serve to add to the certainty that all *a*'s are the 2′-derivatives and all *b*'s the 3′.

(42) W. E. Cohn, in E. Chargaff and J. N. Davidson (eds.), "The Nucleic Acids," Academic Press, Inc., New York, N. Y., in press.

OAK RIDGE, TENNESSEE

[CONTRIBUTION FROM THE LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

The Isolation of Protogen

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Procedures are described for the isolation of protogen, a growth factor for *Tetrahymena geleii* and *Streptococcus faecalis*. The bound form of protogen occurring in liver was liberated by hydrolysis and extracted into chloroform. The extract contained several forms of protogen. The predominant form, Protogen-A, was purified by solvent countercurrent distribution and chromatographic adsorption. Upon mild oxidation Protogen-A was converted into another form, Protogen-B, which was obtained pure by solvent countercurrent distribution and chromatographic adsorption.

In determining the nutritional requirements of *Tetrahymena geleii* Kidder and Dewey[1-3] found that a supplement such as liver extract was required for growth of the organism. The unknown factor in the supplement designated as Factor II[4] was later shown to contain two components, designated IIA and IIB, the latter of which was found to be replaceable by a mixture of pyridoxine and copper.[3] Stokstad, *et al.*,[5] studied the stability and chemical properties of Factor IIA and showed it existed in more than one form and suggested the name protogen in view of its essentiality for the growth of protozoa.

Snell and Broquist[6] compared the stimulatory effect of concentrates of protogen, the "acetate-

(1) G. W. Kidder and V. C. Dewey, *Biol. Bull.*, **87**, 121 (1944).
(2) G. W. Kidder and V. C. Dewey, *Arch. Biochem.*, **8**, 293 (1945).
(3) G. W. Kidder and V. C. Dewey, *ibid.*, **20**, 433 (1949).
(4) V. C. Dewey, *Biol. Bull.*, **87**, 107 (1944).
(5) E. L. R. Stokstad, C. E. Hoffmann, M. A. Regan, D. Fordham and T. H. Jukes, *Arch. Biochem.*, **20**, 75 (1949).
(6) E. E. Snell and H. P. Broquist, *ibid.*, **23**, 326 (1949).

TABLE I

THE ISOLATION OF PROTOGEN FROM BEEF AND PORK LIVER

	Units $\times 10^{-6}$[a]	Solids, g.	Units/mg.
Protogen-B			
1. 4 tons ground fresh liver extracted with hot water. Filtered. Insoluble cake digested with papain at *p*H 5–6, 75°, 36 hr. Filtered, aqueous digest evaporated to tar.			0.6
2. 240 kg. tar (eq. to 4 tons liver) autoclaved 120°, 3.5 hr. in 480 l. of 3.3 *N* NaOH. Cooled, adjusted *p*H 5.5 with concd. HCl. 160 l. ethanol and 420 l. chloroform added. Stirred, chloroform phase separated and reduced to 25 l.	520	1940	268
3. 4 kg. magnesol[b] slurried in chloroform. Filtered, washed twice with 10 l. fresh chloroform. Filtrates combined, reduced to 2 l.	335	956	350
4. Batch divided in half. 40 tube, 80 transfer solvent countercurrent distribution between 0.5 *M* phosphate *p*H 7.2–chloroform 1 l. per phase on each half. Tubes containing Protogen-A peak combined, adjusted to *p*H 3, stirred. Chloroform phase reduced to 300 ml.	75; 100	8.0; 10.2	9,600
5. Chromatographic adsorption of Protogen-A on 6.5 × 65 cm. silicic acid[c] column. Developed with (a) 5 v. chloroform, (b) 2.5 v. 1% methanol in chloroform.[d] Percolates containing Protogen-A from each half of batch combined, reduced to 50 ml.	160	1.24	129,000
6. Chromatographic adsorption of Protogen-A on 3.6 × 52 cm. silicic acid column. Developed with (a) 1.5 v. benzene, (b) 4 v. 5% acetone in benzene. Percolate containing Protogen-A reduced to dryness. Dissolved in 50 ml. chloroform.	107	0.145	740,000
7. Protogen-A converted to Protogen-B with 5% *t*-butyl hydroperoxide in chloroform 26°, 20 hr.	65		
8. 200 tube, 200 transfer solvent countercurrent distribution of Protogen-B between 3% acetic acid in water–chloroform. Tubes containing Protogen-B peak evaporated to dryness. Dissolved in 10 ml. benzene.	52	0.0346	1,500,000
9. Chromatographic adsorption of Protogen-B on 2 × 15 cm. silicic acid/celite 1/1 column. Developed with (a) 1 v. 10% acetone in benzene, (b) 1.5 v. 22% acetone in benzene. Column sectioned and eluted with methanol chloroform 1/1. Eluate containing Protogen-B.	44	0.0246	1,800,000
Protogen-A			
7a. Starting sample, material from step 6. Partition chromatography between 0.5 *M* phosphate *p*H 8 supported on 3.6 × 40 cm. celite 545[e] column and (a) 4 v. ethylene chloride and (b) 2 v. chloroform. Protogen-A band.	240 135	0.302 0.103	795,000 1,310,000
8a. Chromatographic adsorption on 2 × 16 cm. silicic acid column. Developed with 1.8 v. 4% acetone in benzene. Protogen-A band.	91	0.048	1,900,000

[a] One unit of protogen is defined as that amount which will give the same growth for *T. geleii* as one mg. of a papain digest of a water-insoluble fraction of liver.[5] [b] Magnesol Industrial powdered, Westvaco Chemical Division, South Charleston, W. Va. [c] Silicic acid, Merck Reagent Grade. Fine particles removed by water flotation, washed with 6 *N* hydrochloric acid, then acetone and air dried. [d] One v. is volume required to wet adsorbent. [e] Johns-Manville Corp., New York, N. Y.

replacing factor"[7] and the "pyruvate-oxidation factor"[8] on the growth of certain lactic acid bacteria on an acetate-free medium and concluded that the three factors were identical. The similarity in properties of protogen and an unidentified factor required for *Butyribacterium rettgeri* has been pointed out.[9]

The properties and purification of the "acetate-replacing factor,"[7,10,11] the "pyruvate-oxidation factor,"[12,13] and the "*B. rettgeri* factor"[9] have been reported. The "acetate-replacing factor," renamed "α-lipoic acid,"[14] and a derivative of protogen[15] have been obtained in a crystalline state, and DL-6,8-dithioöctanoic acid (6-thioctic acid, DL-α-lipoic acid), a compound with the biological properties of protogen has been synthesized.[16–18] We wish to report the procedures used for the isolation of protogen.

Experimental and Discussion

Assay Method.—The assay for protogen used in the preliminary isolation work was the growth of the protozoan,

(7) B. M. Guirard, E. E. Snell and R. J. Williams, *Arch. Biochem.*, **9**, 381 (1946).

(8) D. J. O'Kane and I. C. Gunsalus, *J. Bact.*, **56**, 499 (1948).

(9) L. Kline and H. A. Barker, *ibid.*, **60**, 349 (1950).

(10) L. J. Reed, B. G. DeBusk, P. M. Johnston and M. E. Getzendaner, *J. Biol. Chem.*, **192**, 851 (1951).

(11) L. J. Reed, M. E. Getzendaner, B. G. DeBusk and P. M. Johnston, *ibid.*, **192**, 859 (1951).

(12) L. J. Reed, I. C. Gunsalus, G. H. F. Schnakenberg, Q. F. Soper, H. E. Boaz, S. F. Kern and T. V. Parke, THIS JOURNAL, **75**, 1267 (1953).

(13) I. C. Gunsalus, L. Struglia and D. J. O'Kane, *J. Biol. Chem.*, **94**, 859 (1952).

(14) L. J. Reed, B. G. DeBusk, I. C. Gunsalus and C. S. Hornberger, Jr., *Science*, **114**, 93 (1951).

(15) E. L. Patterson, J. A. Brockman, Jr., F. P. Day, J. V. Pierce, M. E. Macchi, C. E. Hoffmann, C. T. O. Fong, E. L. R. Stokstad and T. H. Jukes, THIS JOURNAL, **73**, 5919 (1951).

(16) M. W. Bullock, J. A. Brockman, Jr., E. L. Patterson, J. V. Pierce and E. L. R. Stokstad, *ibid.*, **74**, 1868 (1952).

(17) M. W. Bullock, J. A. Brockman, Jr., E. L. Patterson, J. V. Pierce and E. L. R. Stokstad, *ibid.*, **74**, 3455 (1952).

(18) (a) M. W. Bullock, J. A. Brockman, Jr., E. L. Patterson, J. V. Pierce, M. S. von Saltza, F. Sanders and E. L. R. Stokstad, *ibid.*, **76**, 1828 (1954); (b) C. S. Hornberger, Jr., R. F. Heitmiller, I. C. Gunsalus, G. H. F. Schnakenberg and L. J. Reed, *ibid.*, **74**, 2382 (1952); **75**, 1273 (1953).

T. geleii.[5] Later an assay using *Streptococcus faecalis* (A.T.C.C. 8043) on an acetate-free medium containing propionate was adopted.

S. faecalis was carried on a yeast dextrose agar stab. The inoculum was grown at 37° for 24 hours in 10 ml. of the assay medium supplemented with 10 mg. of a papain digest of a water-insoluble liver fraction. The cells were washed once with saline. The assay medium was that described by Broquist and Snell[19] with the following modification. The amino acid mixture was replaced by 200 mg. of enzymatic hydrolyzed casein and 1000 mg. of acid-hydrolyzed casein per 100 ml. of double strength medium.

A pad plate assay was used. To prepare the assay plates final strength medium containing 1.5% agar and 0.25% sodium propionate was autoclaved at 120° for 10 minutes. After cooling to 45°, the medium was inoculated with 1.5 ml. of inoculum per 100 ml. and 200 ml. poured into unsterilized 7.5 × 12 inch Pyrex baking dishes. The dishes were covered with glass plates and stored for use in the chill room for as long as 15 days.

The assay was carried out by pipetting 0.02 ml. of each sample onto 1/4 inch diameter filter paper discs[20] which were placed on the assay plate along with a series of discs making up a standard protogen curve. After incubation at 37° for 16 hours, the growth diameter around each disc was measured and used to calculate the protogen content of the sample from the standard protogen curve.

Starting Material.—The isolation procedures for protogen showing a typical run are outlined in Table I. The starting material was a mixture of beef and pork liver. It was ground and extracted with hot water, and the residue was digested with papain. The protogen activity in the papain digest tar was non-dialyzable and could not be extracted into organic solvents. However, after hydrolysis with either acid or alkali, it was readily extracted from aqueous acid but not alkaline medium by organic solvents such as chloroform, *n*-butanol, isopropyl ether and ethyl acetate. Alkali was preferred to acid for the hydrolysis because complete solution was affected. Upon acidification after hydrolysis a heavy black tar precipitated carrying considerable protogen activity which was not recovered in the chloroform extract. By the addition of one-fourth volume of ethanol complete solution was maintained.

For *T. geleii* the protogen activity was the same before and after hydrolysis of the tar. However, by *S. faecalis* assay the protogen activity increased about threefold during hydrolysis to equal the *T. geleii* activity.

The chloroform extract contained an oily impurity that interfered in subsequent steps. The impurity was removed by adsorption on magnesol. It could be removed also by the less convenient process of precipitation from acetic acid solution with ethyl acetate.

Solvent Countercurrent Distribution.—The protogen activity in the chloroform extract was first purified by solvent countercurrent distribution by the method of Craig.[21] In such a distribution it was observed that the activity distributed into three different forms as shown in Fig. 1.

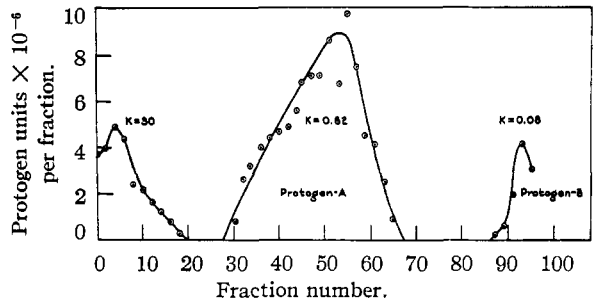

Fig. 1.—Solvent countercurrent distribution of the protogen activity in the chloroform extract of alkaline autoclaved water-insoluble portion of beef liver; system: chloroform 1.0 l., 0.5 *M* phosphate *p*H 7.2, 0.75 l., 40 tubes, 95 transfers.

The predominant form (K = 0.82) was called Protogen-A,[22] and the water-soluble form (K = 0.08) was called Protogen-B.[22] The third form (K = 30) distributed only to a very small extent into water from chloroform. Several solvent systems were satisfactory for the purification of Protogen-A and Protogen-B. Some of these are shown in Table II. Chloroform against aqueous buffer solutions at the proper *p*H formed the most suitable systems for both Protogen-A and Protogen-B from the standpoint of high resolution, large solvent capacity, non-inflammability and minimal emulsion formation.

Chromatographic Adsorption.—Protogen-A and Protogen-B were purified by chromatographic adsorption on silicic acid. Their rates of movement with various developers are shown in Table III.

TABLE II

DISTRIBUTION COEFFICIENTS OF PROTOGEN-A AND PROTOGEN-B

$$\text{Dist. coef. } (K) = \frac{\text{concn. in organic phase}}{\text{concn. in aqueous phase}}$$

System	Dist. coef. Protogen-A	Dist. coef. Protogen-B
Chloroform 0.5 *M* phosphate *p*H 7.2	0.8	0.08
Chloroform 3% acetic acid in water	100	3.0
Methyl isobutyl ketone 0.5 *M* phosphate *p*H 7.2	1.8	0.05
Methyl isobutyl ketone 3% acetic acid in water	18	1.7
Ethyl acetate 0.5 *M* phosphate *p*H 7.2	2.2	0.05
Ethyl acetate 3% acetic acid in water	20	1.4
Toluene 0.5 *M* phosphate *p*H 6.4	0.67	
n-Butanol 0.1 *M* carbonate *p*H 8	12	1.3
n-Butanol 1 *M* acetate *p*H 4.5	18	4.5
Isopropyl ether 0.2 *M* phosphate *p*H 6.6	0.68	0.08
Isopropyl ether 3% acetic acid in water	10	0.3

TABLE III

CHROMATOGRAPHIC ADSORPTION OF PROTOGEN-A AND PROTOGEN-B ON SILICIC ACID[a]

Developer	R_f[b] Protogen-A	R_f[b] Protogen-B
Chloroform	0.04	
1% Methanol in chloroform	.22	
2% Methanol in chloroform		0.27
3% Methanol in chloroform		.33
5% Methanol in chloroform		.66
4% Acetone in benzene	.42	
20% Acetone in benzene		.24
22% Acetone in benzene		.37

[a] Silicic acid, Merck Reagent Grade. Fine particles removed by water flotation, washed with 6 *N* hydrochloric acid, then acetone and air dried. Prewashed according to W. A. Schroeder, *Ann. N. Y. Acad. Sci.*, **49**, 204 (1948). [b] Reciprocal of number of volumes of developer required to move material to bottom of column.

(19) H. P. Broquist and E. E. Snell, *J. Biol. Chem.*, **188**, 431 (1951).

(20) Penicillin assay discs from Carl Schleicher and Schuell Co., New York, N. Y.

(21) See discussion by L. C. Craig and D. Craig on Extraction and Distribution in A. Weissberger, "Technique of Organic Chemistry," Vol. III, Interscience Publishers, Inc., New York, N. Y., 1950, p. 171.

(22) The properties of Protogen-A and of α-lipoic acid[12] suggest the substances are identical. The identity of Protogen-B and β-lipoic acid has been suggested previously.[12]

Relationship between Protogen-A and Protogen-B.—The chemical relationship between Protogen-A and Protogen-B is one of oxidation–reduction as indicated by the equation

$$\text{Protogen-A} \underset{[O]}{\overset{[H]}{\rightleftharpoons}} \text{Protogen-B}$$

Mild oxidizing agents such as *t*-butyl hydroperoxide, air and iodine convert Protogen-A to Protogen-B. The stronger agents bromine, nitric acid and potassium permanganate destroy the biological activity. By reduction with sodium borohydride, or less satisfactorily with zinc and acetic acid, Protogen-B is reduced to Protogen-A. These interconversions are shown in Fig. 2 by means of bioautographs.

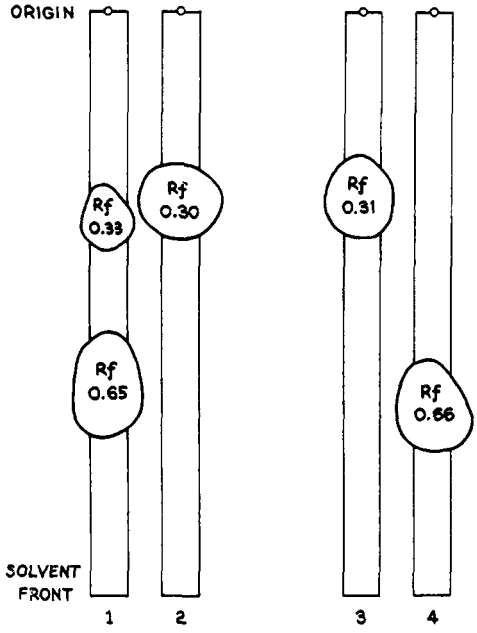

Fig. 2.—Bioautographs showing the interconversions of Protogen-A and Protogen-B developed with *n*-butanol saturated with 0.5 *N* ammonia and assayed with *S. faecalis:* 1, Protogen-A; 2, Protogen-A treated with *t*-butyl hydroperoxide; 3, Protogen-B; 4, Protogen-B treated with sodium borohydride.

Samples of 30–60 protogen units in 0.01 ml. of solution were put on one-half inch wide No. 1 Whatman filter paper strips. The strips were developed by the ascending technique with *n*-butanol saturated with 0.5 *N* ammonia for 6 hours at room temperature and assayed on *S. faecalis* agar plates. The Protogen-B found on strip 1 was not a contaminant but arose from air oxidation while the sample was drying on the paper prior to development. When such strips were cut between the two spots and the portion containing the Protogen-A redeveloped, a Protogen-B spot was again present.

In the isolation procedures as Protogen-A became purified an appreciable amount was lost through oxidation to Protogen-B. The reaction proceeded especially fast in the presence of light or during storage in ether solutions. Therefore, after adsorption on silicic acid Protogen-A was usually deliberately converted to Protogen-B which was then further purified. The conversion was carried out in dilute chloroform solution at room temperature for 20–40 hours with a large excess of *t*-butyl hydroperoxide. No Protogen-A remained at the end of the reaction period, and the recovery of protogen activity as Protogen-B was 74 ± 20%.

Properties of Protogen-A and Protogen-B.—A criterion of purity used to advantage on samples of a few milligrams was a comparison of the protogen activity and oxidizable solids with a theoretical curve in a solvent countercurrent distribution. The solids were determined by a modification of the chromic acid oxidation method of Johnson.[23] The amount of material for a single determination was 15 to 50 γ so that only 0.5 to 1 mg. of the total sample was consumed. Such a comparison is shown in Fig. 3 where Protogen-B was purified by countercurrent distribution in the system ethyl acetate–3% acetic acid in water. In the region in which Protogen-B occurs the biological activity and oxidizable solids were distributed almost exactly according to the theoretical curve for a single substance, indicating the sample was essentially pure. Such samples usually exhibit a faint blue fluorescence which was removed by chromatographic adsorption on silicic acid.

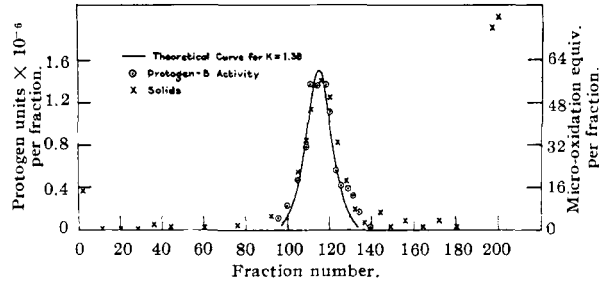

Fig. 3.—Solvent countercurrent distribution of Protogen-B; system ethyl acetate 10 ml., 3% acetic acid in water 10 ml.: —, theoretical curve for $k = 1.38$; O, Protogen-B activity; X, solids.

Protogen-B from the column was a yellow oil soluble in alcohol, acetone, chloroform, sparingly soluble in water and benzene and insoluble in petroleum ether and carbon tetrachloride. The optical activity was $[\alpha]^{25}D$ 105° (*c* 0.94, benzene). Two separate samples prepared by this procedure gave the following analyses: C, 42.14; H, 6.56; S, 26.84; and C, 40.46; H, 6.10; S, 32.36. Protogen-A was obtained by the procedures 1–6, 7a, 8a in Table I as a light yellow oil soluble in alcohol, acetone, chloroform and benzene and insoluble in water and petroleum ether.

A comparison of the infrared curves of Protogen-A and Protogen-B is shown in Fig. 4.

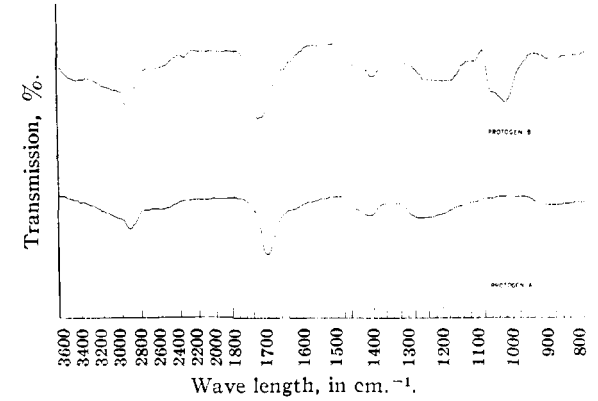

Fig. 4.—Infrared absorption spectra of Protogen-A (bottom curve) and Protogen-B (top curve).

Acknowledgment.—We wish to thank Dr. R. C. Gore, Stamford Laboratories, American Cyanamid Company, for the determination of the infrared absorption curves.

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