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STUDIES ON PRE-BIOLOGICAL EVOLUTION. III. BLACK AZULMIC ACIDS OBTAINED FROM AMMONIUM CYANIDE

M. Labadie, R. Jensen and E. Neuzil

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STUDIES ON PRE-BIOLOGICAL EVOLUTION
III. BLACK AZULMIC ACIDS OBTAINED FROM AMMONIUM CYANIDE

M. Labadie, R. Jensen and E. Neuzil[1]

ABSTRACT. The authors have investigated the formation and some physico chemical properties, and discuss the structure, of black azulmic acids, polymers obtained by heating an aqueous solution of ammonium cyanide. The interest in azulmic acids is connected with the problem of pre-biological molecular evolution; the hydrolysis of azulmic acids gives rise to several natural amino acids, urea, guanidine, glycocyamine and 5-amino-4-imidazole carboxamidine, (abbreviation: AICA).

INTRODUCTION /525*

Approximately 15 years ago, following the work of Miller [1,2] and especially of Oro et al. [3,4] as well as Lowe, Rees and Markham [5], ammonium cyanide and its polymers (including the azulmic acids) stimulated considerable interest in connection with theories of the origin of life and pre-biological molecular evolution.

In reality, azulmic acids have been known for a very long time; in 1806, Proust [6] observed in the course of aging of an aqueous solution of cyanhydric acid, the formation of a black precipitate, called "azulmic acid" by Boullay [7] and later "azulmine" because of its similarity in appearance to peat or "ulmin." In 1869, Guatier [8], who was one of the first to stress the polymer nature of the substance, stated that azulmine is a polymer of HCN, with a formula $(HCN)_9O_3$, with the oxygen supplied by the water. Voelker [9,10] has shown that anionic p polymerization is involved, not influenced by the presence of oxygen [11] and equally capable of taking place in an anhydrous medium [10].

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[1]With the assistance of Miss. A. Mellet, Technical Assistant

Laboratory of Medical Biochemistry, Faculty of Medicine and Pharmacy, Bordeaux, France (Authors' Affiliation)

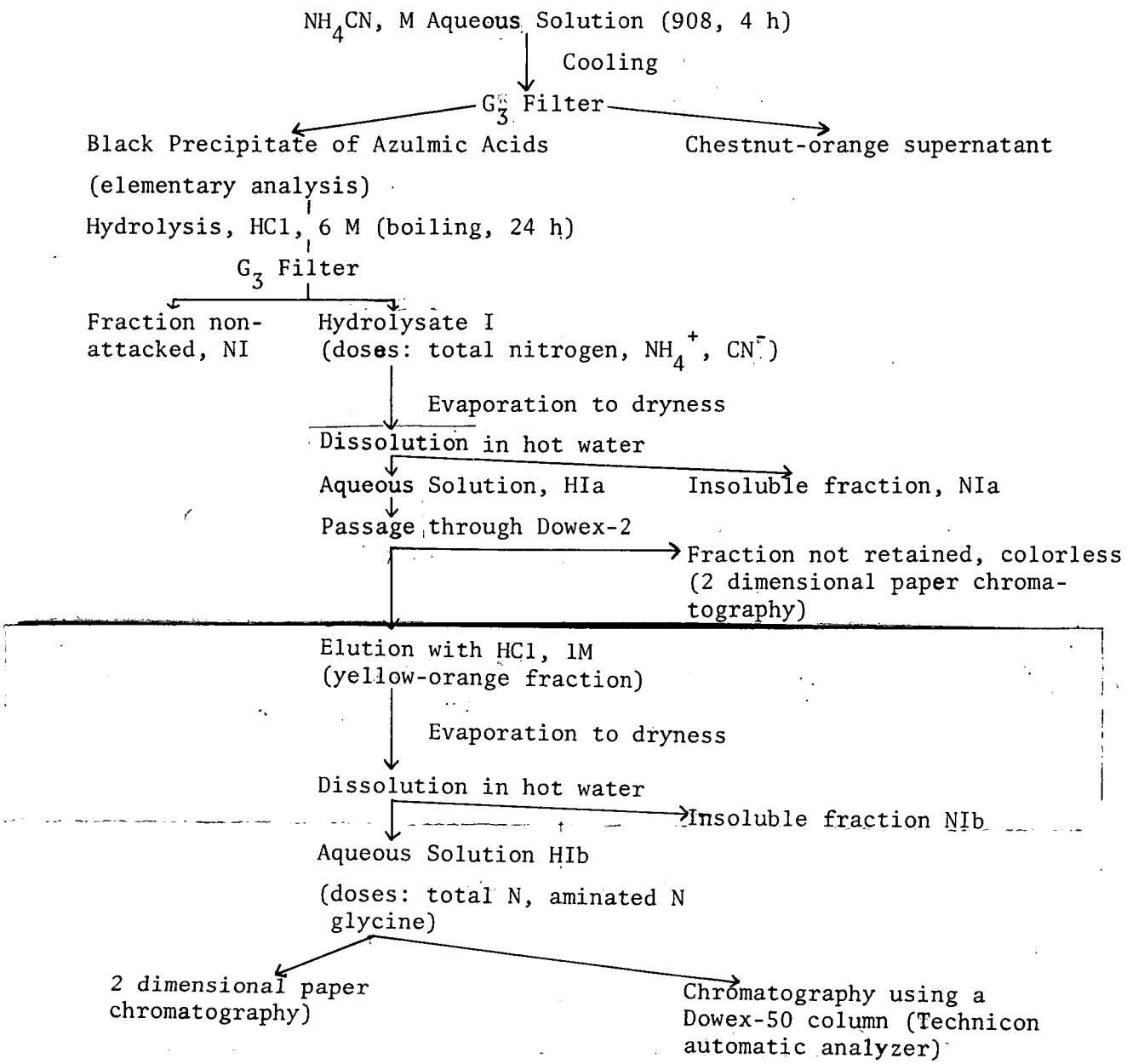
*Numbers in right hand margin indicate pagination in the foreign text.

We ourselves repeated the work of Lowe, Rees and Markham [5] on the nature of pre-biological evolution [12, 13] and we present herein our experimental results concerning the black azulmic acids obtained by heating NH_4CN in an aqueous solution.

MATERIAL AND TECHNIQUES /526

Preparation of Ammonium Cyanide and Black Azulmic Acids

A 1 M solution of ammonium cyanide was prepared by extemporaneously neutralizing an aqueous solution of HCN with NH_4OH, with the HCN obtained from gaseous HCN [14]. This solution (100 ml) was heated for a few hours at a constant temperature in a glass vessel with mechanical agitation; after cooling, the black polymers [15] were collected on a filter with G_3 porosity, eluted with distilled water, dried, then subjected to hydrochloric hydrolysis (method I) or other types of hydrolysis (see the experimental results section).



Method I. Separation Followed by Hydrochloric Hydrolysis of Black Polymers Obtained from an Aqueous Solution of NH_4CN, 1M.

Doses

The CN^- ions were measured using argentimetry [16] and the NH_4^- ions using the method of Fleury and Loth [17], while the total nitrogen was determined using the method of Grabar [18] using Markham's apparatus [19]. The aminated nitrogen and glycine were determined respectively using the methods outlined in [20,21]. Urea was determined by means of diacetylmonoxime [22].

Chromatographic Analysis

(1) Chromatography using an ion exchange resin column.

The fractioning of the hydrolysates of the black azulmic acids was carried out using Dowex-2 X8 resin (200-400 mesh in the form of HO^-) in a /527 glass column (10 cm x 1 cm). The determination of amino acids was performed using Dowex-50 X4 resin (column: 130 cm x 0.6 cm) using the Technicon auto-analyzer (Chauncey).

(2) Two-dimensional paper chromatography.

The two fractions of the hydrolysate, separated following passage through Dowex-resin, were subjected to two-dimensional chromatography using Whatman 3MM paper. The solvent phases used are given in the following list: Phase I, *n*-butanol-acetic acid-water (12:3:5, v/v/v); Phase II, phenol: 1000 g, water: 250 ml, NH_4OH (d = 0.88): 6 ml; Phase III, *n*-butanol-pyridine-water (1:1:1, v/v/v); Phase IV, methanol-90% formic acid-water (16:3:1, v/v/v); Phase V, *n*-butanol-water (172:28, v/v).

The chromatograms were studied under ultraviolet light (λ = 254 mμ) or treated with various indicated reagents: acetonic solutions of ninhydrin (0.5%) or isatine (0.2%) for the amino acids; Ehrlich's reagent for urea; diacetyl [23] or ferricyanide-nitroprussate [23] reagent for guanidine and its derivatives; Pauly's reagent [23] for the imidazole derivatives.

Certain spots were cut out and then eluted by allowing them to remain for 20 minutes at 60° in 5 ml HCl (0.1 M); after cooling, we measured their absorption spectra both in 0.1 M HCl and 0.1 M NaOH (UNICAM SP 800 recording spectrophotometer).

5-Amino-4-Imidazole-Carboxamidine

The 5-amino-4-imidazole-carboxamidine (AICA) was prepared in the form of a dichlorohydrate by acid hydrolysis of adenine [24] (hydrate of the hemi-sulfate, Lab Cyclo). The recrystallized product, still containing traces of adenine and hypoxanthine, was transformed into a chromatographically pure base (R_F = 0.63, IV; R_F = 0.10, V; R_F = 0.52, III; R_F = 0.40, I; λ_{max} (HCl: 0.1 M) = 282 mμ; λ_{max} (NaOH 0.1 M) = 292 mμ) by passing its aqueous solution through Dowex-2 which retains the impurities; in the chromatograms, the AICA spot is visible, being colored bluish-violet in ultraviolet light, showing up as yellow when treated with Ehrlich's reagent, violet when treated with the ferricyanide-nitroprussate reagent, purplish-gray in response to the diacetyl reagent, garnet when treated with Pauly's reagent and orange when treated with isatine.

EXPERIMENTAL RESULTS

Conditions of Formation of Black Azulmic Acids

(1) Influence of Temperature and Heating Time.

After several hours, even at room temperature, an aqueous solution of NH_4CN which is initially colorless becomes yellowish-orange as a precipitate of black azulmic acids forms gradually. Following various heating times, followed by storage for several hours in a refrigerator, the black precipitate is collected on a filter; at a given temperature (Table 1), the weight of the precipitate increases with heating time, tending toward a maximum value; on the other hand, for a given heating time, the weight increases with the temperature in the range from 70-90°; we have been unable to study temperatures above 90° because of the condensation of NH_4CN on the cold parts of the vessel in the form of yellowish lumps.

For the majority of our later tests we adopted a heating regime of four hours at 90°.

Table 1. Influence of Temperature and Heating Time on the Weight of Black Azulmic Acid Formed from a Molar Aqueous Solution of NH_4CN (100 ml). /528

	Heating for 4 Hours			Heating at 90°			
	70°	80°	90°	2h	4h	8h	16h
Weight of black azulmic acids(g)	0.380	0.590	0.814	0.550	0.814	0.910	0.960
Yield (%) (calculated relative to C)	12.29	19.06	26.30	17.77	26.30	29.40	31.02

(2) Influence of pH of the reaction medium.

The reaction mixture has an initial pH of 9.58, with this value increasing slightly in the course of heating at 90° to reach a value of 10.00 at the end of the fourth hour. At pH values below 8 or above 10.5, the reaction mixture heated under the same conditions remains practically colorless; the optimum yield of black azulmic acids corresponds to an initial pH of 9.58.

The black azulmic acids appear in the form of an amorphous black powder which is not hygroscopic (C: 38.77; H: 3.96; N: 40.85; O: 16.42%), with a melting point above 350°C. This powder, which is very poorly soluble in water and the classical organic solvents, is quite soluble, however, in soda, formic acid and concentrated sulfuric acid and especially in dimethylsulfoxide, a less agressive solvent dispite is oxidizing properties [25]. The solution of black azulmic acids in dimethylsulfoxide at 0.2 mg/ml, studied in a cuvette 1 ml thick, shows an absorption maximum at λ = 252 mμ (pH 9).

Chemical Analysis

By virtue of the polymer nature of the product, we hydrolyzed it in order to determine the constituents. The black azulmic acids which were obtained after heating for four hours at 90° were hydrolyzed by HCl or by $Ba(OH)_2$. Finally, we studied the action of dinitrofluorobenzene and determined the amount of $-NH_2$ groups by polarography.

(1) Chlorhydric Hydrolysis

The black azulmic acids (0.814 g) were subjected to reflux boiling in HCl, 6 M, for 24 hours (Method 1); at the end of this hydrolysis, 57.8% of the blackish NI substance still remained undissolved in the HCl relative to the initial weight; this insoluble residue was separated by filtration; the filtrate (hydrolysate I) had a musty orange color: N total: 13 matoms; NH_4^+: 9.8 mions; CN^-: 0.65 mions; N total - ($N_{NH_4^+}$ + N_{CN^-}): 2.55 matoms.

This filtrate was then evaporated to dryness; 0.822 g of clear chestnut powder were collected. After dissolution in 40 ml of hot water (60 mg remain insoluble: NIa fraction), this HIa hydrolysate is passed through Dowex-2 resin, which allows us to separate two fractions: (a) a fraction which is not retained, alkaline, colorless, eluted by water, which, following concentration at reduced pressure, is subjected to two-dimensional paper chromatography with solvent phases I-V. The appearance of these chromatograms, following examination in ultraviolet light and pulverization of the reagents (Pauly, diacetyl, ferricyanide-nitroprussate), is shown in Figure 1.

We were able to characterize the AICA (A), a compound previously identified by Oro and Kimball [26] in the supernatant, guanidine (G), glycocyamine (Y) and urea (U). The spectrophotometric dosage of AICA following elution and the dose of urea have respective values of 8.6 and 39 micromoles for 822 mg of hydrolysate I. /529

(b) A fraction eluted by HCl, 1 M[1], containing several amino acids: this eluate, with a yellowish-orange color, after evaporation yields a dry residue weighing 0.197 g; this residue is in turn dissolved in 20 ml of hot water (here again a fraction remains insoluble, the NIb fraction with a weight of 10 mg); using this HIb solution, we perform two-dimensional paper chromatography using several pairs of solvent phases: (1) with the pair previously used, we obtained chromatograms showing about 10 spots that could be revealed using Pauly's reagent; among the latter, there was a T spot which was very significant and appeared bluish-violet in color when viewed under ultraviolet light, orange as revealed by Pauly's reagent, and whose R_F in the I system

[1]Following elution with HCl, 1M, there was still a chestnut zone fixed in the resin, which was impossible to elute.

is equal to 0.44 and 0.33 in the V system; this spot, after elution, shows an absorption maximum at λ = 256 mμ (in HCl, 0.1 M) and 2 maxima at λ 260 and 287 mμ in NaOH (0.1 M); (2) with pairs I and III or II, we were able to identify seven amino acids (spots 1-7) out of 11 ninhydrin-positive spots (Figure 2): glycine, γ-amino-butyric acid, aspartic acid[1], alanine, histidine, threonine, and serine. The spot corresponding to the glycine was by far the most important; moreover, there were two spots (8 and 9) which showed up as pale bluish-violet when treated with ninhydrin and two spots (10 and T) which were revealed as yellow with the same reagent was employed; the latter spot corresponds exactly to the T spot previously described; its R_F in solvent phase III is equal to 0.74 and 0.70 in the II solvent phase. Moreover, there are several spots that are visible under ultraviolet light.

In the HIb solution, we also found the amount of total nitrogen, that of aminated nitrogen as well as that of glycine. Finally, we submitted this solution to chromatographic analysis using Dowex-50 resin (Table II).

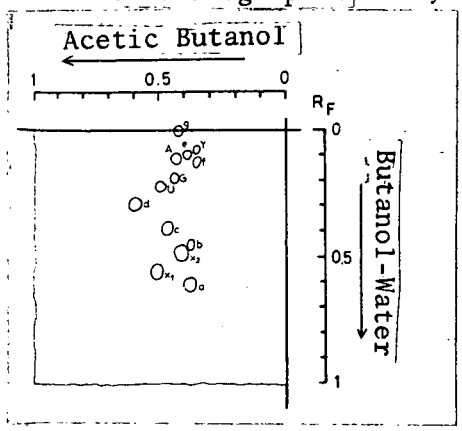


Figure 1. Two-dimensional Paper Chromatography of the Fraction Which Is Not Retained by Dowex-2 Resin of the Hydrolysate of Black Azulmic Acids.

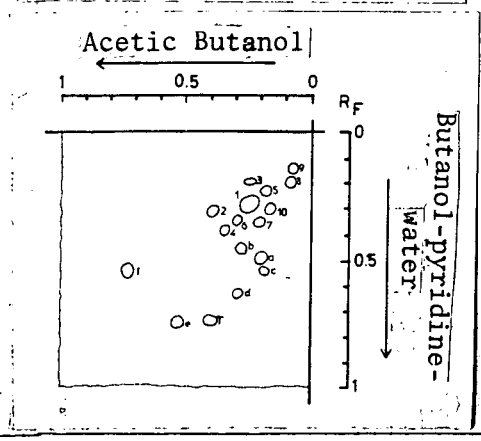


Figure 2. Two-dimensional Paper Chromatography of the HIb Fraction Eluted from Dowex-2 Resin by HCl, 1M.

[1]Spot 3 is blue after treatment with isatine.

Table II. Substances Identified by Chromatography Using Dowex-50 Resin and Determined in the Eluate of HCl, Fraction HIb, of the Hydrolysate of Black Polymers.

Three supplementary ninhydrin-positive compounds were not identified; v_1 is eluted before aspartic acid, v_2 and v_3 are eluted at the end, after histidine. The results are expressed in µatoms, µions, and µmoles depending on the particular case.

Substances	Results for the total HIb solution
Total nitrogen	1845
Aminated nitrogen	787
NH_4^+	40
Glycine	594
γ-amino-butyric acid	16
Aspartic acid	15
Alanine	8
Histidine	1.6
Threonine	<1
Serine	<1
Lysine	<1
Ornithine	<1
Urea	traces
v_1	2.6
v_2	13
v_3	4

We also performed a careful chlorhydric hydrolysis: the black azulmic acids were heated to 100° for seven hours in HCl, 6M.

The fraction which was not retained by the Dowex-2 resin then contained only 4.5 µmoles of AICA, with the HIb fraction containing smaller quantities of each amino acid relative to those of Table II: 109 µmoles for glycine, 1.65 µmoles for aspartic acid, 0.043 µmole for alanine.

(2) Hydrolysis by Baryte.

A new sample of black azulmic acid (0.814 g) was subjected to reflux boiling in an aqueous solutin of Ba $(OH)_2$, 0.1 M, for 24 hours; after cooling,

an insoluble blackish residue was removed by filtration; it was abundant and, after being washed and dried, represented 71% of the initial weight; the filtrate, following elimination of the baryte and concentration, was then subjected to chromatographic analysis using paper and a Dowex-50 column; the barytic hydrolysate appeared to be much poorer in compounds of biological significance than the chlorhydric hydrolysate, because we were able to find glycine (52 μmoles), alanine (10.9 μmoles), urea (0.3 μmole) and guanidine.

(3) Tests to Reveal Free Aminated Groups.

Using black azulmic acids, reduced to finely divided powder, we determined the $-NH_2$ groupings by polarography and studied the action of 2, 4-dinitrofluorobenzene.

(a) Amount of groups $-NH_2$. We used the polarographic method described by Jensen and Laroude [27]; 814 mg of black azulmic acids yield 525 μatoms of aminated nitrogen.

(b) Effect of 2, 4-dinitrofluorobenzene. Inasmuch as the black azulmic acids are insoluble in water, we used the Sanger [28] technique as applied in a heterogeneous medium for the insoluble scleroproteins according to Biserte et al. [29]. Following chlorhydric hydrolysis, no DNP-amino acid was liberated by the black azulmic acids.

DISCUSSION /531

The azulmic acids appear to us to constitute a complex group of molecules whose degree of polymerization and structure can vary, especially as a function of the mode of formation. In the case of the black azulmic acids prepared according to the conditions which are most similar to ours and correspond to the inorganic formulas given above, Voelker [10] proposed a polypyridinic structure (Figure 3) whose transverse axis is composed exclusively of C-C bonds.

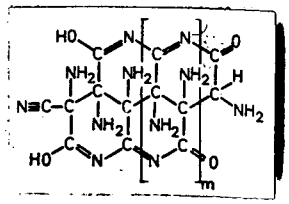
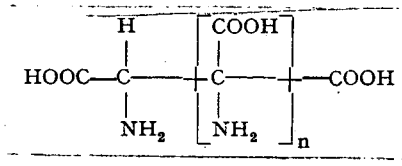


Figure 3. Structure of Black Azulmic Acids According to Voelker [10] (m Being an Odd Whole Number Equal to At Least 7).

In solution in the dimethylsulfoxide, these nodes are therefore responsible for the maximum absorption at $\lambda \cong 252$ mμ[2]. Some of the results of hydrolysis of the black azulmic acids by an acid or a base [30] are also similar to the structure shown in Figure 3, since the carbonized chain is found in products of hydrolysis with a general formula:



In our view, it is difficult to interpret the formation of amino acids described here in the course of hydrolysis by $Ba(OH)_2$ and *a fortiori* by HCl; if the structure proposed by Voelker is correct, we must admit the possibility of a breakage of certain C-C bonds, i.e., a cracking which (primarily in the chlorhydric medium) would yield free radicals capable of recombining in order to merge with the amino acids and the imidazole derivatives; our experimental results are quite similar to those of Lowe, Rees, and Markham [5] as far as the nature and proportions of the amino acids liberated by the chlorhydric hydrolysis of black azulmic acids prepared under conditions slightly different from our own. Within the polymers, we have shown [12] that the amino acids do not appear to be linked by peptide chains: in effect, trypsin has no affect on these polymers; moreover, the infrared absorption spectrum[3] merely shows clearly that the C = O bond, in other words the reaction of the biuret exerted on the solution of polymers in dimethylsulfoxide, is negative[4]. Note finally that the diffusion spectrum of the X-rays by black azulmic acids in powdered form, according to the Debye-Scherrer method, showed a ring corresponding to a period of 3.28 Å, the same spectrum that was found in the NI fraction that was evidently not attacked by chlorhydric hydrolysis.

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[2] We have verified that the spectrum of pyridine in solution in dimethylsulfoxide (cuvette 1 mm thick) shows this maximum, but there is also a maximum at λ=258mμ.

[3] In solution in dimethylsulfoxide, black azulmic acids have an infrared spectrum which is difficult to interpret due to the presence of the bonds of the solvent.

[4] We reassured ourselves that dimethylsulfoxide does not itself disturb this action.

It appears that azulmic acids constitute important stages in the pre--biological molecular evolution; as a matter of fact, these polymers form spontaneously from very simple molecules and their hydrolysis liberates substances which are of biological significance: urea, guanidine, glycocyamine, amino acids and derivatives of imidazole including histidine and AICA, which is an immediate precursor of adenine; finally, the chestnut azulmic acids which are soluble in the supernatant and are separated by Lowe, Rees and Markham [5] into several electrophoretic fractions have the curious property of gradually becoming insoluble in the form of microspherules which are morphologically quite similar to those of coccic bacteria [12,13]. /532

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