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The Effect of Various Parameters on the Rate of Formation of Fibers from Collagen Solutions[1]

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The influence of several parameters on the rate of fiber formation from collagen solutions has been determined by measuring the increase in turbidity as the gel was formed. The ranges of the environmental factors were as follows: temperature, 28–37.5°; *p*H, 7.1–8.5; ionic strength, 0.085–0.115. The order of decreasing inhibition (increasing rate) at constant ionic strength for anions is $S_2O_3^{=}$, $SO_4^{=}$, Cl^-, Br^-, I^-, CNS^- and for the cations, Ba^{++}, Ca^{++}, Mg^{++}, Li^+, Na^+, K^+. The maximum rate for the reaction is found to occur near *p*H 8. The ionic strength or type of salt has little effect on the optimum *p*H despite the fact that the mobility of the protein is greatly affected by these factors. The energy of activation for the reaction is unchanged by the type of cation or by the concentration of the salt. It is altered slightly by *p*H and urea, greatly by different anions.

Although it has been generally recognized that many factors, such as *p*H, ionic strength, type of salt and temperature, will influence the formation of fibers from solutions of collagen,[3–6] little has been done to study all of them systematically. Randall, *et al.*,[7] have made a preliminary report on a systematic study. Their method, as with the others, was to note the end product of fiber formation either grossly or in the electron microscope. Gross[8] has reported a few results obtained by following the reaction during fiber formation. It is the purpose of this research to conduct such a systematic study by measuring the kinetics of collagen formation.

Materials and Methods

Preparation of Collagen Solutions.—Our method of preparation combines certain elements of the procedures described by Gross[9,10] and by Jackson and Fessler.[4] The skin of either a hairless, unborn calf or a very young calf was obtained fresh from the abattoir. It was immediately placed in a cold room set at 1°. All subsequent operations were performed at this temperature. The flesh side was scraped to remove all adhering fat and muscle. Hair, when present, was shaved off with small-animal clippers. The skin was then ground finely in a meat chopper. Each volume of ground tissue was extracted for 18–20 hr. with twice its volume of 0.1 *M* NaCl. The suspension was filtered through several layers of cheesecloth and the liquid discarded. The tissue was re-extracted with twice its volume of 0.1 *M* HOAc for 24 hr. The extract was removed as before and saved. If the extract appeared turbid, it was clarified by centrifugation. The extract was neutralized with 6 *M* NaOH. NaCl was quickly added to give a final concentration of 18% (wt./vol.), and the mixture was stirred for 18–24 hr. The gel-like precipitate which formed was removed by centrifugation, suspended in a volume of water sufficient to make it fluid and then lyophilized. The dried powder was distributed in small vials and stored at −10°. To prepare the final collagen solutions, a sufficient amount of dried material was dissolved in 0.1 *M* NaCl to give a final protein concentration of 0.10–0.15%. The solution was then dialyzed twice against 0.2 *M* tris-(hydroxymethyl)-aminomethane (tris) buffer, *p*H 8.1, containing 0.05 *M* NaCl. The final ionic strength was 0.15. The solution was clarified by centrifugation at 12,000 × *g* for 30 minutes prior to use.

Recently we have found that soluble collagen can be extracted from acetone-dried skin using either 0.1 *M* neutral phosphate or HOAc solutions. The ground skin was dehydrated after the NaCl extraction by suspending it in 4–5 changes of acetone at 1°. The acetone was removed by filtration and finally by evaporation. Thirty ml. of 0.1 *M* HOAc was used to extract each gram of this dried preparation. The remaining steps of the preparation were carried out in a manner identical to that described for the HOAc extracts of fresh tissue.

All water used in this work was purified by passing distilled water through a column of mixed, ion-exchange resins.

Purity of Protein Preparations.—In Table I are listed the results of various determinations on four samples which had been prepared by slightly different methods. Preparations C2-2 and C4 were derived from the fresh and the acetone-dried skin, respectively. Samples C3 and C2-1 were reprecipitated with 18% NaCl. However, the skin used for C2-1 was not first extracted with 0.1 *M* NaCl.

TABLE I

SUMMARY OF DATA RELATING TO THE PURITY OF THE COLLAGEN PREPARATIONS

Prepn.	(OHPro/N) × 100%[a]	% OHPro after coagulation[b]	% N after coagulation[b]	E_{255}[c]/E_{275}	E_{275}
C2-1	74–84	0	5	0.80	1.67
C2-2	80–91	0	9	.93	2.58
C-3	87–100	0	0	.87	0.90
C-4	78–89	0	2	1.06	1.14

[a] This value is obtained by dividing the protein concentration as calculated from hydroxyproline concentrations by the protein concentration as calculated from nitrogen determinations. The amount of nitrogen in collagen was taken as 18%.[11,12] The double entry is used since the results obtained will depend on whether one assumes a value of 14%[12] or 12.3%[11] for the hydroxyproline concentration of bovine skin collagen. [b] These columns refer to the % of the original amount remaining after coagulation. [c] Ratio of extinction coefficients (for a 1% solution of protein) taken at 255 and 275 mμ.

The amount of hydroxyproline[13] per unit of nitrogen[14] seems to be the best criterion of collagen purity. However,

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(2) Robert J. Frackelton Laboratories, Benjamin Rose Hospital, Cleveland 6, Ohio.

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(13) Determined by the method of C. J. Martin and A. E. Axelrod, *Proc. Soc. Exptl. Biol. Med.*, **83**, 461 (1953).

(14) As determined by digestion followed by Nesslerization.

according to other criteria, our preparations were much purer than this test would indicate. As seen in Table I, all hydroxyproline was removed from solution after fiber formation. The small amount of nitrogen in solution after coagulation indicates that our preparations were 90–100% pure if there were no co-precipitation. Carefully purified rat-tail collagen was found to have a flat portion in the curve of ultraviolet absorption between 257.5 and 287.5 mμ.[15] Our curves were nearly flat in this region showing little or no contamination by proteins containing chromophoric amino acids. The deviation could be represented by the ratio of the extinction coefficient found at the minimum (255 mμ) to that at the maximum (275 mμ) absorption.[16] The differences in the maximum extinction coefficient may be explained by light scattering.

The electrophoretic patterns, obtained using the Perkin–Elmer Model 38A apparatus, differed only slightly from preparation to preparation. A typical pattern is shown in Fig. 1 for the protein at a *p*H near its isoelectric point. The secondary peak appeared only on the descending-limb pattern. It is composed of material that is lost from solution during fiber formation. The nature of this second peak is now being investigated.

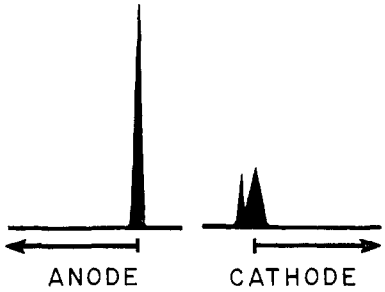

Fig. 1.—Electrophoretic pattern of soluble collagen. Buffer was 0.2 *M* tris with 0.05 *M* NaCl, *p*H 8.3, Γ/2 0.15. Electrophoresis was carried out at 11 mamp. for 225 min. at 1°.

Ultracentrifugal diagrams obtained from solutions containing no more than 0.15% protein showed a single, hypersharp peak. The uncorrected sedimentation rate was 1.1 S. in 0.1 *M* phosphate buffer, *p*H 6.8.

Measurement of Fiber Formation.—The increase in turbidity produced by the formation of fibers from the clear collagen solution formed the basis for our kinetic assay. The gels obtained were of uniform turbidity and contained no particulate matter. This method had been used to determine fibrin formation[17] as well as collagen formation.[8] The apparatus consisted of a Beckman DU spectrophotometer equipped with a double set of thermospacers through which water from a constant temperature bath was circulated. In a typical experiment, 1 ml. of water, 0.7 ml. of buffer and 0.3 ml. of a dilution of the salt to be tested were placed in Wasserman tubes and preincubated for 15 minutes in the water-bath which was set at approximately 28°. At zero time, 1 ml. of the cold protein solution was added. The mixture was incubated in the water-bath for 3 minutes and was then poured into a Corex cuvette which had been pre-warmed by placing it in the instrument. Beginning at 4 minutes the optical density at 290 mμ was read at one-minute intervals.

The curves shown in Fig. 2 are typical of those we have obtained by this method. In order to quantify the rate of reaction, it was necessary to define an end-point. It will be noted that the shape of the curve is a straight line during the maximum rate of increase in optical density. We have chosen as the end-point the time corresponding to the last point on the straight line, read to the nearest half minute. The rate is then inversely proportional to that time.

A statistical analysis of identical determinations showed that the average standard deviation within a group was 0.56 minute, if each group consisted of all determinations completed during the course of a day. However, the standard deviation was 3.8 minutes, if a group consisted of experiments performed on several consecutive days. For this reason, we have endeavored to complete a set of experiments in one day. This frequently forced us to limit the number of determinations. If it were not desirable to do so, we would use an internal standard with each run and express the rate relative to that standard. This method was used in determining the effect of various anions and cations on the rate of fiber formation.

In order to demonstrate that we were obtaining collagen fibers, we took electron micrographs of the final clot. We consistently obtained fibers with the typical 600–700 Å. spacing of collagen.

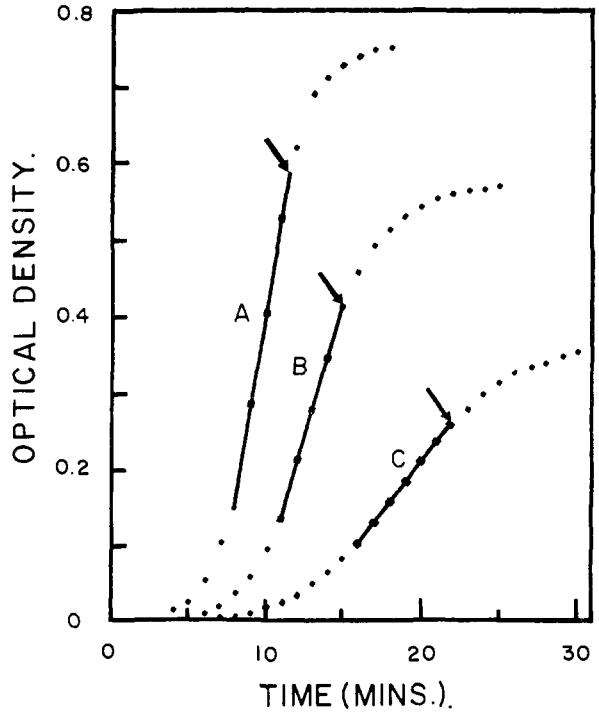

Fig. 2.—Sample determination of optical density *vs.* time using different concentrations of protein. Protein concentrations were: curve A, 0.022%; curve B, 0.016%; curve C, 0.010%.

Results

The Effect of Salts on Coagulation.—The results given in Fig. 3 clearly show that there was an inhibition of the rate of fiber formation with increasing ionic strength in the range indicated. In addition, the specific ion used had an effect. Surprisingly, the order of decreasing effect, in this case a decreasing inhibition, exactly reversed the Hofmeister series for anions. However, in the case of cations, Fig. 4, the order of decreasing effect exactly followed the lyotropic series for the *swelling* of gelatin.[18]

With Cu and Zn salts, a gel which could be dissolved in Versene was formed even at 1°. Examination in the electron microscope showed the gel to be amorphous.

Binding of Ions by Soluble Collagen.—Electrophoretic mobility studies of the protein in the presence of various salts was used to determine the extent of ion binding. Unfortunately, electrochemical considerations make electrophoresis im-

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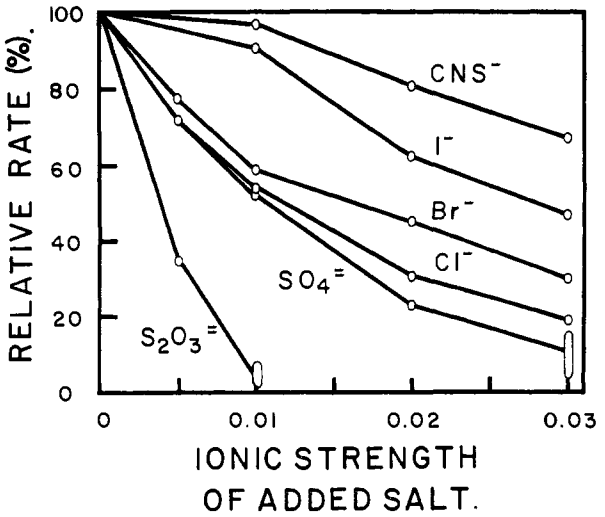

Fig. 3.—The effect of various anions at increasing ionic strength on the rate of fiber formation. The basic environment contained tris buffer at a final ionic strength of 0.085. Sodium was the cation in all cases.

possible using AgCl electrodes in the presence of CNS^-, I^- or Br^-.

The mobility of the primary peak of the soluble collagen in different salts is given in Table II. It can be seen that the collagen was more anionic in the presence of $SO_4^=$ than in Cl^-, and more cationic in the presence of Ba^{++} than in Na^+. Thus it is evident that, in this case, $SO_4^=$ and Ba^{++} were bound to a greater extent than Cl^- and Na^+, respectively. To extrapolate from these experiments, it appears that the effect of anions on coagulation follows the order of the extent of binding.

TABLE II

THE EFFECT OF VARIOUS IONS ON THE MOBILITY OF SOLUBLE COLLAGEN IN TRIS BUFFER AT *p*H 8.3

Salt added	Ionic strength Salt	Ionic strength Buffer	Mobility × 10⁵ Asc.	Mobility × 10⁵ Desc.
None	..	0.10	....	+0.17
NaCl[a]	0.05	.10	.00	0.00
NaCl	.10	.10	− .18	− .18
NaCl[a]	.05	.10	.00	.00
$BaCl_2$	.05	.10	+ .27	+ .28
Na_2SO_4	.05	.10	− .21	− .20
None	..	.15	+ .19	+ .19
NaCl	.05	.10	.00	.00
NaCl	.10	.05	− .19	− .16
$BaCl_2$	.05	.10	....	+ .27
$BaCl_2$	.10	.05	....	+ .45

[a] These results are from the same determination.

As indicated by the results in Table II, increasing the concentration of NaCl made the collagen more anionic. This fact demonstrates that Cl^- was bound to a greater extent than Na^+. When the amount of tris buffer was increased with a concomitant decrease of NaCl, the protein became more cationic. However, when the tris buffer was increased relative to $BaCl_2$, the protein became less cationic. Thus, it appears that the order of decreasing binding is Ba^{++}, $tris^+$, Na^+.

The Effect of *p*H.—In these experiments the protein was dialyzed against 0.15 *M* NaCl. The

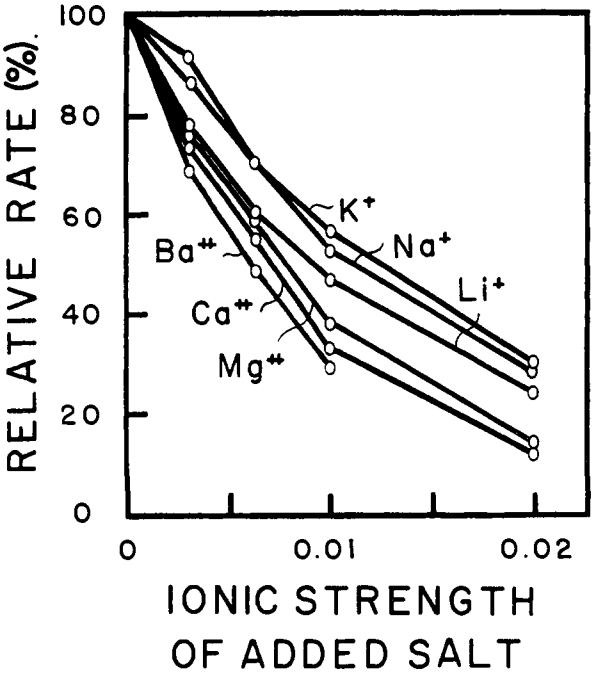

Fig. 4.—The effect of various cations at increasing ionic strength on the rate of fiber formation. The basic environment contained tris buffer at a final ionic strength of 0.085. Chloride was the anion in all cases.

ionic strength of the tris buffers was kept constant by using 0.15 *M* HCl while varying the amount of tris. The final mixture then consisted of 1.0 ml. of water, 0.7 ml. of buffer at the appropriate *p*H, 0.3 ml. of the salt to be tested and 1.0 ml. of the protein solution.

As seen in Table III, there was a small apparent shift in the optimum *p*H with increasing inhibition. Nevertheless, the rates at the higher ionic strengths were considerably decreased even at the optimum *p*H. In addition, the optimum was shifted to lower values with increasing ionic strength irrespective of the salt used. This seemed inconsistent, since, as indicated in Table II, the concentration and type of salt used had a marked effect on the isoelectric point of the protein. Thus it appears that the *p*H optimum is not determined by the isoelectric point.

As seen in Table IV, tris buffer inhibited the reaction in much the same manner as the inorganic salts. For this reason we believe that the tris cation acts as an inhibitor. It is obvious that the $tris^+$ is a more active inhibitor than Na^+. It should, therefore, be pointed out that the quantitative results found in our experiments would probably not be duplicated in another buffer system, particularly if the anion were the important buffer ion.

We have found that there is a decrease in rate without the concomitant shift in optimum *p*H with decreasing protein concentration or increasing urea concentration. Thus it appears that a decrease in rate is not always accompanied by a shift in the *p*H optimum.

The Effect of Temperature.—In Figs. 5 through 7 are given plots of the Arrhenius equation for the

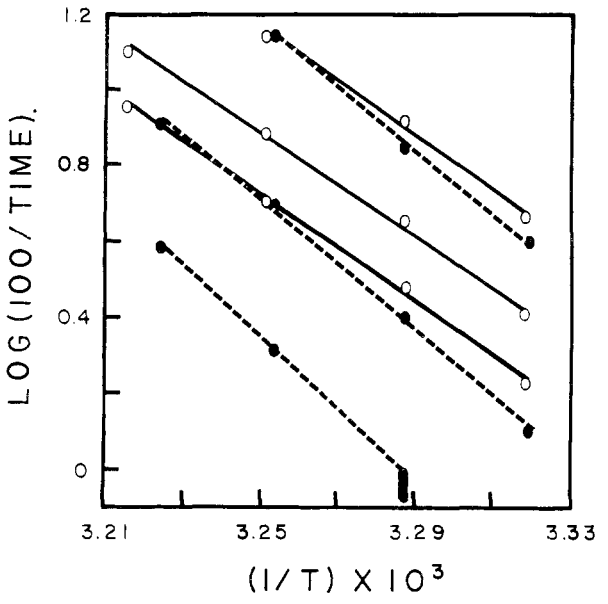

Fig. 5.—(a) The effect of increasing concentrations of urea on the energy of activation. Reading from top to bottom for the broken lines, the concentrations were 0.11, 0.22 and 0.33 *M*. (b) The effect of increasing concentrations of NaCl on the energy of activation. Reading from top to bottom for the solid lines, the concentrations were 0.01, 0.02 and 0.03 *M*.

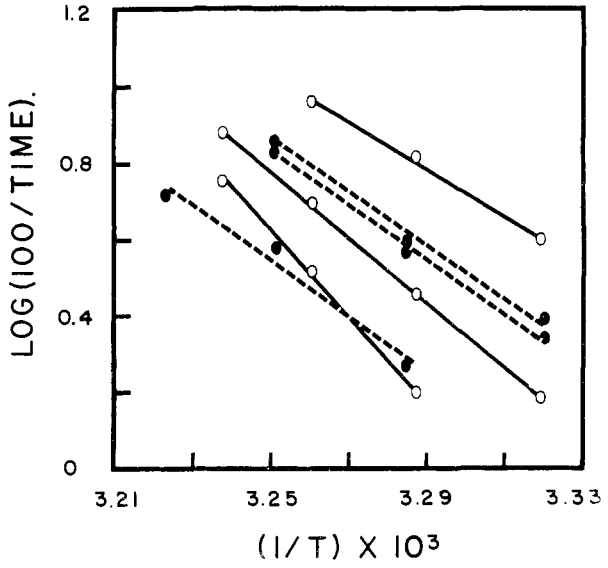

Fig. 6.—The effect of anions and cations on the energy of activation. (a) Reading from top to bottom for the broken lines, the cations were Na^+, Li^+ and Ba^{++}. (b) Reading from top to bottom for the solid lines, the anions were I^-, Cl^- and $SO_4^=$.

effect of temperature on the rate of coagulation under varying experimental conditions. A most striking result is that certain conditions which changed the rate of coagulation did not alter the slope of the straight line. The activation energies calculated from the slopes in Figs. 5b (solid lines) and 6a (broken lines) ranged from 32–35 kcal. There was a larger range of activation energies (23–35 kcal.) found on varying the *p*H in the pres-

TABLE III

EFFECT OF SALTS ON THE OPTIMUM *p*H OF FIBER FORMATION

Salt	Ionic[a] strength	*p*H	Rate (100/time)	Opt. *p*H
NaCl		8.5	9.5	
	0	8.1	10.5	8.1
		7.7	9.5	
		7.1	4.5	
		8.5	5.0	
		8.1	6.3	
	0.01	7.7	6.3	7.9
		7.1	3.2	
		8.5	2.9	
		8.1	3.5	
	.02	7.7	3.7	7.7
		7.1	2.6	
Na_2SO_4		8.5	8.3	
		8.1	10.0	
	0	7.7	7.7	8.2
		7.3	6.3	
		8.5	3.8	
		8.1	4.5	
	0.008	7.7	4.4	7.9
		7.3	4.2	
		8.5	1.7	
	.015	8.1	2.3	≦7.3
		7.7	3.1	
		7.3	3.3	
$BaCl_2$		8.5	5.7	
	0	8.1	6.7	7.9
		7.7	6.7	
		7.3	5.6	
		8.5	2.2	
		8.1	3.2	
	0.008	7.7	3.6	7.6
		7.3	3.3	
		8.5	..	
		8.1	1.6	
	.015	7.7	1.8	≦7.3
		7.3	2.0	

[a] This represents the ionic strength contribution of the added salt only. The ionic strength of the buffer present in each case was 0.085.

TABLE IV

EFFECT OF VARIOUS RATIOS OF TRIS BUFFER TO SODIUM CHLORIDE ON THE OPTIMUM *p*H OF FIBER FORMATION

Ionic strength Tris buffer	NaCl	*p*H	Rate (100/time)	Opt. *p*H
		8.5	10.5	
0.03	0.07	8.2	9.1	≧8.5
		7.7	6.9	
		7.3	5.7	
		8.5	7.7	
		8.2	8.0	
.05	0.05	7.7	6.3	8.2
		7.3	5.4	
		8.5	2.0	
		8.2	3.7	
.1	0	7.7	2.8	8.0
		7.3	..	

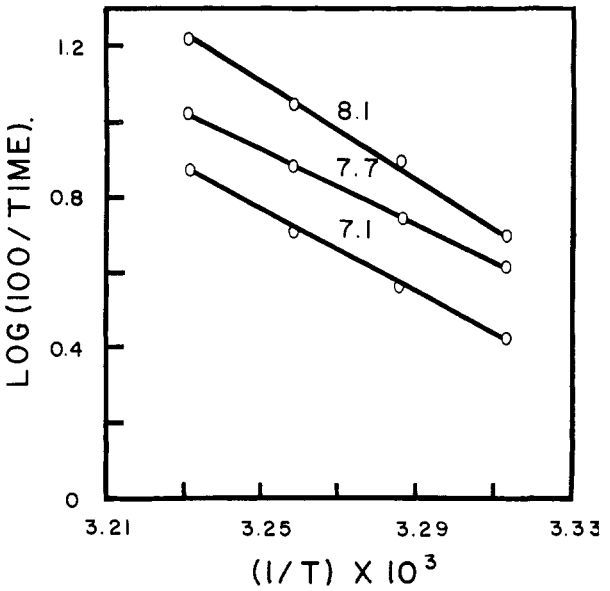

Fig. 7.—The effect of varying *p*H on the energy of activation.

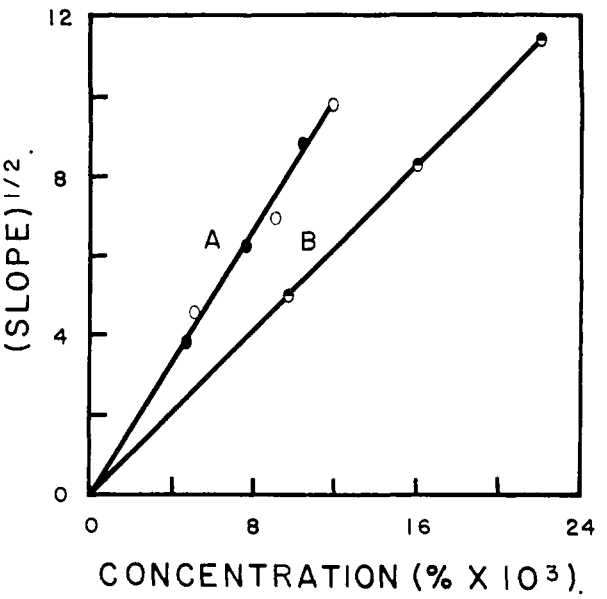

Fig. 8.—The correlation between protein concentration and the maximum rate of optical density increase. The slope is (optical density/time) × 10^3. Line A was obtained from two separate determinations. Line B was from one experiment at a different ionic strength.

ence of Cl^-. However, repeated attempts failed to bring out any clear relationship between activation energy and *p*H or optimum *p*H. Figure 7 shows one example. The lack of correlation is probably due to the fact that the lag time and the rate of increase in optical density seem to have different optima, as discussed in the next section. In the presence of urea, Fig. 5a (broken lines), there appeared to be a slight increase in the activation energy with increasing concentration: 38, 40 and 45 kcal. at 0.11, 0.22 and 0.33 *M* urea, respectively. It is evident from Fig. 6b (solid lines) that the activation energy is markedly dependent upon the anion used. The values of the activation energies varied from 25 for I^- to 51 kcal. for $SO_4^=$.

Kinetics of the Reaction.—We have attempted to explore the kinetic mechanism of the reaction. Three observations are pertinent. (1) The most obvious is that there was an initial lag period. This was followed by a period of rapidly increasing optical density, the rate of which was consistently constant throughout a major portion of the reaction. This immediately eliminates the probability of a simple, one-step reaction. (2) As seen in Fig. 8, there was an excellent straight-line relationship between protein concentration and square root of the maximum slope of the time–optical density curve. This observation probably eliminates a type of reaction in which a first- or second-order reaction is followed by a first- or second-order reaction. Reactions of this type have a more complex relationship between rate and substrate concentration.[19] (3) Under most experimental conditions the rates of the lag period and the period of increasing optical density were parallel. However, on varying the *p*H of the medium, the two portions appeared to have slightly different *p*H optima. For example, at two different *p*H values, one curve had a slower starting time and a faster slope. This finding was consistent. Thus, it appears that there are two separate and consecutive reactions which are influenced by the environment in a similar fashion. We suspect that there is an initial reaction which is completed, or nearly so, before the second one begins. The major portion of the second reaction then proceeds by second-order kinetics under the influence of a constantly renewed supply of protein, as explained in the next section.

(19) J. Chien, THIS JOURNAL, **70**, 2256 (1948).

Discussion

From the results, it is apparent that increasing the ionic strength inhibits the rate of fiber formation. Since increasing the ionic strength does not change the energy of activation, we may conclude that the inhibition of the rate is brought about by an effective decrease in the concentration of the precipitable protein. However, we have found that all the protein is precipitated at the end of the reaction. Therefore, the inactivated protein must be in equilibrium with the active form. From these observations we postulate that an active group or groups on the molecule complex reversibly with the salt ions to form a product which is incapable of condensing with other molecules to form the collagen fiber. Then, as the unbound molecules are removed from solution, the equilibrium is shifted toward the formation of more unbound protein until all of the protein has precipitated. This may account for the straight-line portion of our optical density–time curve, as discussed in the section on kinetics. The fact that certain ions inhibit more than others may be explained in part by assuming different equilibrium constants for the ion–protein complex, as is indicated by our electrophoretic studies. However, since the energy of activation for fiber formation changes with different anions, another factor must be taken into account. This factor may be due to the binding of a limited number of anions to sites not immediately involved in fiber formation. Such binding could

affect an electrostatic repulsion dependent upon the charge density of the complexing anion. In order to conform to the observation that increasing the ionic strength of the same salt does not affect the energy of activation, we would have to postulate that these sites are fully saturated at low salt concentrations.

The following scheme is the summary of the proposed mechanism of collagen formation and its reversal

$$T' \rightleftharpoons T \rightleftharpoons T^* \rightleftharpoons \text{Collagen}$$

where T is tropocollagen, T′, the inactive form and T*, the proposed intermediate that is being formed during the lag period. T′ is formed when active sites on tropocollagen are blocked by anions, cations or by such agents as urea. Conditions which enhance hydrogen bonding favor the formation of collagen, while those which rupture hydrogen bonds reverse the equilibrium. The equilibrium rate will be influenced by *p*H, type and ionic strength of the salt present, concentration of the protein and temperature. Thus, when the collagen solution is removed from the presence of the fiber and conditions are adjusted to favor collagen formation, the system will remain poised unless conditions also favor a reasonable rate of reaction.

In writing this paper, we have avoided the use of such terms as neutral-soluble or acid-soluble collagen. The impressions gained from this work have cast considerable doubt in our minds as to whether such differences really exist. It must be realized that when HOAc solutions of collagen are adjusted to values above *p*H 7, the optimum *p*H of fiber formation is being approached. If the other variables are such as to favor coagulation, fiber formation will occur. If not, a collagen solution which is soluble in neutral or alkaline solutions will be obtained. We have found that after coagulation, the fibers can be redissolved in HOAc, the collagen reneutralized, and fibril formation repeated as before.

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[CONTRIBUTION FROM THE NATIONAL RESEARCH COUNCIL OF CANADA, PRAIRIE REGIONAL LABORATORY]

Infrared Studies on Complexes of Mg^{++} with Adenosine Phosphates[1]

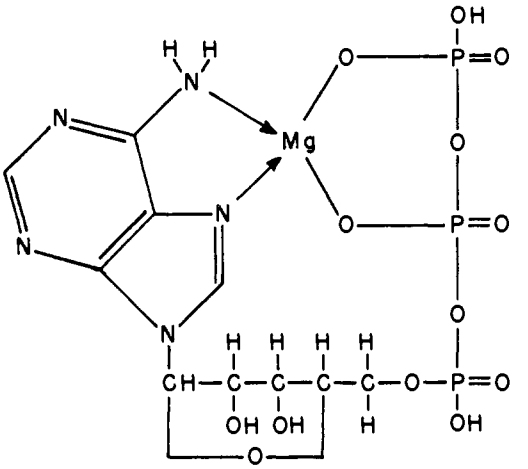
BY AGNES EPP, T. RAMASARMA[2] AND L. R. WETTER

RECEIVED AUGUST 5, 1957

Infrared spectra were obtained for the mono-, di- and triphosphate of adenosine and inosine in the presence and in the absence of Mg^{++}. The presence of the divalent cation affected the pyrophosphate group and the purine nucleus of the di- and triphosphates of adenosine. The data suggest that these divalent ions complex with the above groups but no direct evidence is obtained as to whether the complex is intramolecular or intermolecular.

Introduction

Certain inorganic cations, notably Mg^{++}, are essential for enzymic transphosphorylations involving the adenylic acid system,[3] and a complex of Mg^{++} and ATP[4] is the substrate for many kinases.[5–7] It has been suggested[8] that complexes of this type involve the cation and the pyrophosphate group. Recently, however, Szent-Györgyi[9] has postulated a quadridentate chelate between ATP and Mg^{++}, as in I, in which the bonding involves the 6-amino group, the nitrogen atom at position 7 and one oxygen atom of each of the terminal phosphate groups.



Such a chelate would be expected to give a characteristic infrared absorption spectrum distinct from that of uncomplexed ATP. Infrared spectra of nucleic acids have been recorded earlier[10–17] and

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(4) The following abbreviations are used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5′-monophosphate; ITP, inosinetriphosphate; IDP, inosinediphosphate and IMP, inosinemonophosphate.

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