The Effect of Ions on the Kinetics of Formation and the Stability of the Collagen-Fold*

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The effects of a variety of electrolytes on the formation and stability of the collagen-fold have been studied by optical rotatory measurements on dilute solutions of ichthyocol and calfskin gelatins. For all salts, the initial rate of mutarotation (r) following "quenching" of a warm gelatin solution to a temperature below the gelatin $\rightleftarrows$ collagen-fold transition temperature has been found to be related to the concentration of added salt (m) by the relation: $\log (r/r_0) = k_0 m$. Also it has been shown that the temperature of the midpoint of the gelatin $\rightleftarrows$ collagen-fold phase transition (T_m) is directly proportional to salt concentration; *i.e.*, $T_m = T_m^0 = Km$. Thus the effects of various neutral salts may conveniently be described in terms of the constants k_0 and K. The kinetic parameter, k_0, constitutes a measure of the effect of a salt on the nucleation of the gelatin $\rightleftarrows$ collagen-fold phase transition, while the equilibrium parameter, K, serves to characterize the effect of salts on the stability of the equilibrium collagen-type helix. Comparison with results obtained by others with concentrated gelatin gels shows that specific ion-binding to the gelatin chain is *not* responsible for the effects observed. The data suggest that ions exert their effects through a competitive reorganization of water molecules structurally involved in the formation and stabilization of the collagen-fold.

The striking effects of certain neutral salts on the structure of collagen fibers and gelatin gels have been studied by many investigators (*e.g.*, see Ferry, 1948; Gustavson, 1956). Yet, despite the effort and care invested and the large volume of data gathered, the molecular basis of the neutral (or lyotropic) salt effect has proved remarkably elusive, largely because the molecular structure of collagen itself had not been determined when the majority of these studies were carried out. However, within the last few years general agreement has been reached on the main features of the conformational structure of collagen (Rich and Crick, 1955, 1961; Cowan *et al.*, 1955; Ramachandran, 1956; Bear, 1956) and some progress has been made toward an understanding of the mechanism of formation and stabilization of the collagen-fold from random coil gelatin. In particular, the central role of the pyrrolidine ring in controlling and stabilizing the collagen structure has been appreciated (Harrington, 1958; von Hippel and Harrington, 1959, 1960; Burge and Hynes, 1959; Piez, 1960; Flory and Weaver, 1960), and the results of detailed conformational studies of poly-L-proline (Harrington and Sela, 1958; Steinberg *et al.*, 1960) have been applied to the collagen problem. These developments have been reviewed recently (Harrington and von Hippel, 1961a).

Recent studies on the development of the collagen-fold after cooling of gelatin to temperatures below the collagen $\rightleftarrows$ gelatin transition temperature have suggested that solvation of the backbone carbonyl oxygens might be involved in stabilizing the poly-L-proline II-type helix characteristic of the individual chains of collagen (von Hippel and Harrington, 1960; Harrington and von Hippel, 1961b). And this, coupled with the many earlier suggestions that water might somehow be involved in the structure of collagen (see Harrington and von Hippel, 1961a) has induced us to examine the effects of specific salts on the formation of the collagen-fold in order to see whether these electrolytes might be operating upon the protein via interactions with the proposed structural water. The results of this study seem indeed to suggest that neutral salts affect the collagen-fold by competitively reorganizing the water involved in stabilizing the helix.

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Materials and Methods

Gelatin.—Both the ichthyocol and calfskin gelatins used were kindly provided to us as purified, lyophilized collagen preparations by Dr. P. M. Gallop of the Albert Einstein College of Medicine. The preparation of these collagens from carp swim-bladders and calfskin has been described previously (Gallop, 1955; Seifter *et al.*, 1959). The lyophilized collagen was dissolved and simultaneously converted to gelatin by stirring for about 30 minutes at 50° in 0.025 M $CaCl_2$ (*p*H 7). The resulting slightly turbid gelatin solutions were freed of undissolved residues by centrifuging at 20,000 × g for 60 minutes at 40°, followed by filtration through a type HA (0.45 μ pores) Millipore filter if necessary. Protein

concentrations were determined by the modified Lowry (Folin-biuret) procedure previously described (Gellert *et al.*, 1959) and also by polarimetry at 40°. The specific rotation of a given gelatin in a specific solvent at a fixed temperature above the gelatin ⇄ collagen-fold transition temperature is very constant, and affords a rapid and accurate method for measuring gelatin concentrations. For ichthyocol gelatin in 0.025 M $CaCl_2$ (*p*H 7), $[\alpha]_{313}^{40°} = -692°$, while $[\alpha]_{313}^{40°}$ for calfskin gelatin under the same conditions is $-743°$.

Salts.—The salts and other reagents used were all Analytical Reagent grade and were used without further purification. Hygroscopic salts were dried over P_2O_5 before solutions were made up.

Optical Rotation.—The polarimetric measurements were made with a Rudolph Model 80 Precision Polarimeter equipped with photoelectric attachment and oscillating polarizer. The light source was a Hanovia Quartz Mercury Burner, and most of the measurements were made at the 313 mμ Hg line. The solutions were held in thermostated 1-decimeter tubes equipped with silica (UV transmitting) end-plates. Cell temperatures were controlled to ±0.05°. The temperature in the sample tube was determined (by linear interpolation) by measuring the temperatures in two dummy tubes inserted on either side of the sample tube in the line carrying the circulating coolant. At low temperatures the polarimeter tube end-plates were kept free of condensate by passing a continuous stream of dry air through the polarimeter trough assembly.

Melting curves were obtained by transferring the cold sample into the precooled polarimeter tube and then raising the temperature in 3 to 4° steps, holding at each temperature until the rotation changed by less than 0.002° in 30 minutes. At temperatures in the transition region, times as long as 18 hours were sometimes required to get this close to equilibrium.

Initial rates of mutarotation were measured by pipetting heated (40°) samples of the gelatin-salt mixtures to be tested into precooled polarimeter tubes with one end-plate removed. The end-plate was then rapidly replaced and measurements were begun within 1 minute after the transfer. The inner bore of the tube used was only 3 mm and the total capacity only 0.7 ml, so the sample reached thermal equilibrium almost instantly. Measurements were made at 1-minute intervals for 30 minutes after quenching, and the initial rate was determined by extrapolating back to zero time. Initial rates of up to 0.05° minute could be measured in this way to better than ±5% accuracy.

Results

Most previous studies on the effect of neutral salts on gelatin have used the temperature of macroscopic melting of the solid gel network, formed in the presence of the salt to be tested, as a primary comparative measure of the effect of a series of specific salts. However, in order to focus primarily on intramolecular parameters and to minimize possible effects of intermolecular interactions and aggregation, the studies to be described were carried out at protein concentrations well below the gelation level (mostly at concentrations below 2 mg/ml). Thus our first problem was to establish one or more parameters which might serve as quantitative measures of the molar effectiveness of a particular salt in altering the properties of the collagen-type helix in dilute solution. With this objective, we began a detailed investigation of the effect of varying concentrations of calcium chloride on both the kinetic and the equilibrium properties of the collagen-fold.

Our earlier studies on the formation of the collagen-fold had been carried out in neutral 0.5 M $CaCl_2$, initially because this concentration of neutral salt was needed to solubilize native collagen (Gallop *et al.*, 1957a) and because the collagenase employed in the "enzymatic-probe" studies (von Hippel and Harrington, 1959; von Hippel *et al.*, 1960) is a calcium-activated enzyme (Gallop *et al.*, 1957b) and thereafter in order to maintain a constant, and therefore comparable, ionic environment. However, both the kinetics of formation of the collagen-fold and the stability of the equilibrium structure are strongly dependent upon the $CaCl_2$ concentration.

In Figure 1 the initial rate of mutarotation, after "quenching" of a warm (40°) sample of ichthyocol gelatin to 4°, is plotted as a function of molarity of $CaCl_2$. Clearly, the initial rate in 0.5 M $CaCl_2$ is about eight times less than that attained in very dilute salt. Figure 2 shows the magnitude of the total change in specific levorotation ($\Delta[\alpha]_{313}$) for the same gelatin samples after long standing at low temperature (44 days at 5°). It may be seen that the total increase in specific levorotation (and thus the equilibrium amount of helix formed) is essentially independent of $CaCl_2$ concentration, at least at levels below approximately 0.5 M. Thus the rate differences shown in Figure 1 reflect actual changes in the kinetics of approach to the end-point of the reaction, rather than changes in the equilibrium fraction of protein destined to go into the helical form. Even the apparent decrease in ($\Delta[\alpha]_{313}$) at very high concentrations of $CaCl_2$ may not be real, since at these salt concentrations the mutarotatory process has been slowed so much that even 44 days at 5° may not be sufficient to permit final equilibrium to be attained.

The effect of high and low *p*H is also examined in Figures 1 and 2, and it is clear that both $(d[\alpha]/dt)_0$ and $\Delta[\alpha]$ are essentially independent of hydrogen ion concentration, except for the marked drop in initial rate observed at *p*H 2.5 and low $CaCl_2$ concentration (Fig. 1). However, it should be noted that $\Delta[\alpha]_{313}$ for these

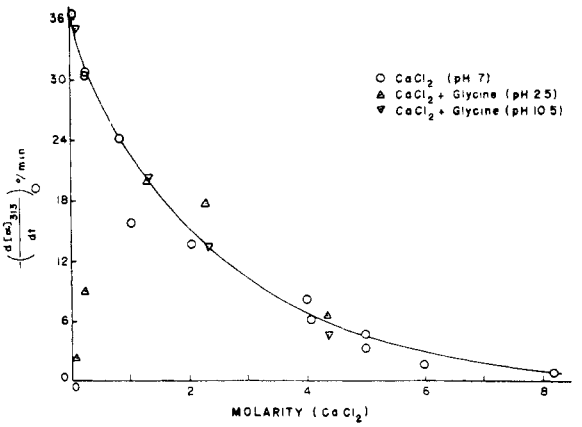

FIG. 1.—Initial rate of mutarotation of ichthyocol gelatin at 4° as a function of $CaCl_2$ concentration. Quenched from 40° to 4° at zero time. Protein conc. = 1.56 mg/ml; ⊙, $CaCl_2$ (*p*H 7); △, $CaCl_2$ + 0.02 M glycine (*p*H 2.5); ▽, $CaCl_2$ + 0.02 M glycine (*p*H 10.5).

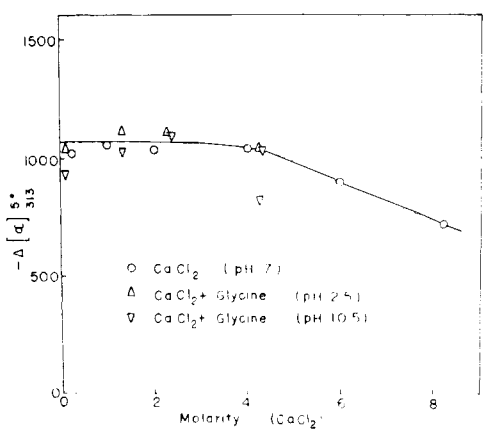

FIG. 2.—Total change in specific rotation of ichthyocol gelatin samples as a function of $CaCl_2$ concentration, after 44 days at 5°. Protein conc. = 1.56 mg/ml; ⊙, $CaCl_2$ (*p*H 7); △, $CaCl_2$ + 0.02 M glycine (*p*H 2.5); ▽, $CaCl_2$ + 0.02 M glycine (*p*H 10.5).

points is essentially unaffected (Fig. 2).

Since the general shape of the curve in Figure 1 suggests an exponential dependence of $(d[\alpha]/dt)_0$ on $CaCl_2$ concentration, these data are replotted in Figure 3 as log $(d[\alpha]/dt)_0$ versus molarity of $CaCl_2$. It is clear that all the initial rate data over the entire range of salt concentration (with the exception of the previously noted low *p*H–low salt points) fall onto a straight line described by equation (1), where m = salt con-

$$\log r = \log r_0 + k_0 m \qquad (1)$$

centration (in moles/liter), $r = (d[\alpha]/dt)_0$, $r_0 = (d[\alpha]/dt)_0$ extrapolated to $m = 0$, and k_0 = the slope (in liters/mole) of the best straight line through the experimental points.

Figure 3 suggests that the constant k_0 might provide a useful comparative measure of the

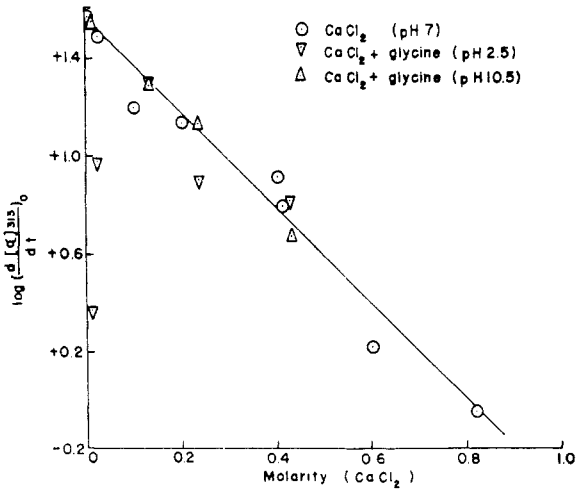

FIG. 3.—Logarithm of initial rate of mutarotation of ichthyocol gelatin at 4° as a function of $CaCl_2$ concentration. Quenched from 40° to 4° at zero time. Protein conc. = 1.56 mg/ml; ⊙, $CaCl_2$ (*p*H 7); ▽, $CaCl_2$ + 0.02 M glycine (*p*H 2.5); △, $CaCl_2$ + 0.02 M glycine (*p*H 10.5).

effect of various salts on the kinetics of formation of the collagen-fold, provided that the linear dependence of log $(d[\alpha]/dt)_0$ on m is a general phenomenon. As will be seen below (Figure 6 and Table I), such plots have indeed been shown to be linear for all salts tested.

Previous comparative experiments on the rate of helix formation in H_2O and D_2O (von Hippel and Harrington, 1960; Harrington and von Hippel, 1961b) showed that solvent alterations leading to an increase in the initial rate of mutarotation at a given temperature seemed to be associated with an increase in T_m (the melting temperature of the protein-solvent system concerned), suggesting that in the present case the gelatin ⇄ collagen-fold transition temperature might also vary with $CaCl_2$ concentration. Figure 4 presents melting profiles for ichthyocol gelatin solutions (containing various concentrations of $CaCl_2$) which had been held at 5° for 24 hours before the beginning of the experiment. Clearly T_m (defined here as the midpoint of the transition) decreased progressively with increasing molarity of $CaCl_2$. Values of T_m derived from these melting profiles are plotted as a function of $CaCl_2$ concentration in Figure 5. These data may be fitted to a straight line defined by equation (2),

$$T_m = T_m^0 + Km \qquad (2)$$

where T_m = the melting temperature at salt concentration m, and $T_m^0 = T_m$ extrapolated to $m = 0$. The slope $K\left(\text{in } \frac{°C}{\text{mole/liter}}\right)$ provides a measure of the molar effectiveness of a given salt in altering the gelatin ⇄ collagen-fold transition temperature, and thus also the stability of the over-all helical structure in that particular ionic environment. Comparison of the low-

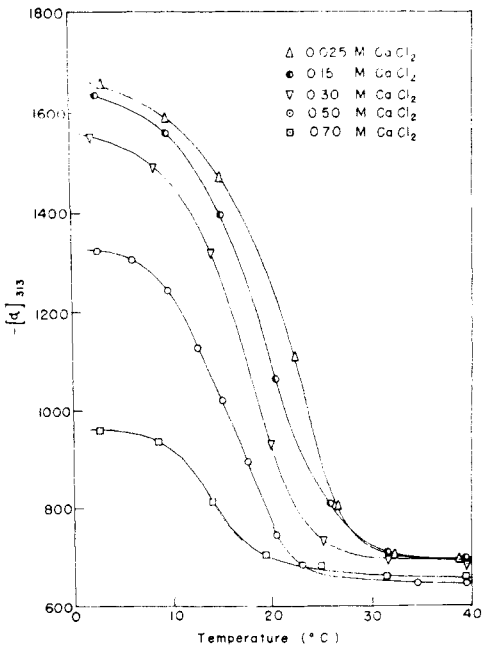

FIG. 4.—Specific rotation of samples of ichthyocol gelatin at various $CaCl_2$ concentrations as a function of temperature, after 24 hours at 5°; *p*H 7; protein conc. = 1.6 mg/ml; △, 0.025 M $CaCl_2$; ◐, 0.15 M $CaCl_2$; ▽, 0.30 M $CaCl_2$; ⊙, 0.50 M $CaCl_2$; □, 0.70 M $CaCl_2$.

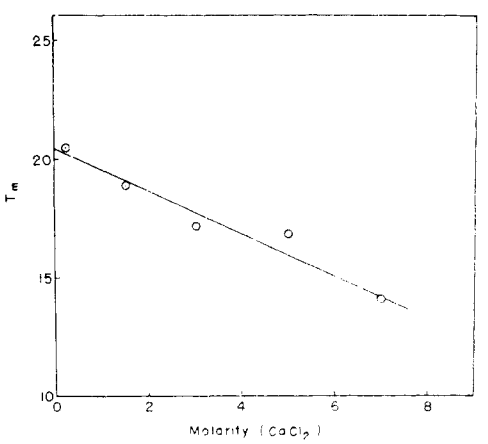

FIG. 5.—T_m (temperature at the midpoints of the melting profiles shown in Fig. 4) for ichthyocol gelatin as a function of $CaCl_2$ concentration.

temperature end of the melting profiles in Figure 4 with values of $\Delta[\alpha]_{313}$ after 44 days at 5° (Fig. 2) reveals that the samples at higher $CaCl_2$ concentration had not reached their equilibrium rotations when melting was begun. However, experiments on a series of gelatin samples in 0.5 M $CaCl_2$, carried through melting experiments after standing at 5° for up to 28 days, showed that the measured T_m is relatively unaffected by long-term changes because the over-all proportions of the melting curve remain essentially constant (see Figure 6, Harrington and von Hippel, 1961b). Hence all samples to be carried through a melting curve for the determination of T_m were routinely incubated in advance for 24 hours at 5°.

After this examination of the gelatin-$CaCl_2$ system, we investigated the effects of a variety of other salts on the initial rate of helix formation and the melting temperature of ichthyocol gelatin. The electrolytes varied markedly in their effect on the collagen-fold, but in each case log $(d[\alpha]/dt)_0$ could be plotted as a linear function of salt concentration, and hence each salt-gelatin system could be assigned a characteristic k_0. Figure 6

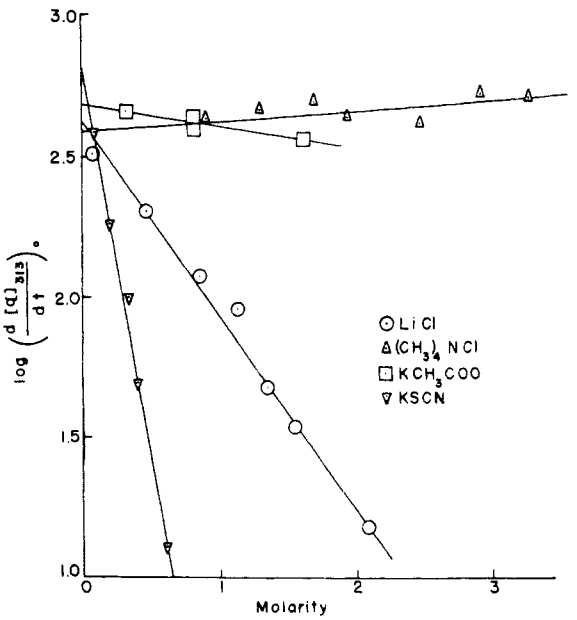

FIG. 6.—Logarithm of initial rate of mutarotation of ichthyocol gelatin at 3° as a function of molarity of added salt. *p*H 7; protein conc. ≃ 1.2 mg/ml; ▽, KSCN; ⊙, LiCl; □, KCH_3COO; △, $(CH_3)_4NCl$.

shows such logarithmic plots for four representative salts with widely different k_0 values; values of k_0 for all the salts tested are collected in Table I.

Figure 6 and Table I show that certain salts, such as $BaCl_2$ and KSCN, substantially reduce $(d[\alpha]/dt)_0$ and are therefore characterized by large negative values of k_0, whereas others, such as KCH_3COO, $(NH_4)_2SO_4$ and $(CH_3)_4NCl$, have relatively little effect and small (positive or negative) k_0 values. All the salts were tested across essentially their entire solubility range. Though some (characterized by large negative values of k_0) entirely prevented collagen-fold formation at high salt concentrations, only two precipitated (or "salted-out") gelatin from solution. These were $(NH_4)_2SO_4$ at concentrations exceeding 0.8 M, and KCH_3COO at concentrations in excess of ~ 3 M. It should be noted that both of these salts are characterized by very small values of k_0, and positive or slightly negative values of K.

Pairs of melting curves, at high and low salt

TABLE I
KINETIC AND EQUILIBRIUM PARAMETERS DESCRIBING THE EFFECTS OF VARIOUS SALTS ON COLLAGEN-FOLD FORMATION IN ICHTHYOCOL GELATIN[a]

Salt	K[b] $\left(\frac{°C}{\text{moles/liter}}\right)$	k_0[c] (liters/mole)
$(NH_4)_2SO_4$	+3.8	+0.12
$(CH_3)_4NCl$	+2.2	+0.04
$(CH_3)_4NBr$	−0.4	−0.08
KCH_3COO	−0.8	−0.08
NH_4Cl	—	−0.28
RbCl	—	−0.37
KCl	−1.4	−0.42
NaCl	−1.6	−0.41
CsCl	−1.8	−0.52
LiCl	−4.1	−0.66
KBr	—	−0.71
KNO_3	—	−1.03
$MgCl_2$	—	−1.07
$CaCl_2$	−8.8	−1.82
KSCN	−10.0	−2.85
$BaCl_2$	—	−3.23

[a] Gelatin concentrations ranged from 1.0 to 1.4 mg/ml; *p*H 7; all samples were 0.005 M with respect to $CaCl_2$ in addition to the other salts added. The k_0 values were derived from mutarotation experiments at ~3°. k_0 is defined by equation (1), K by equation (2). [b] Average estimated error, ±0.5 $\frac{°C}{\text{moles/liter}}$. [c] Average estimated error, ±0.05 liters/mole.

concentrations, were run on a number of the electrolytes listed in Table I. Some representative sets of such curves are shown in Figure 7. T_m values were derived from these curves and K values calculated for each electrolyte tested, assuming in each case a linear dependence of T_m on molarity of salt. These values of K are also tabulated in Table I.

It is important to muster the evidence which demonstrates that T_m *is* linearly related to m for all salts, since this was shown directly in this study only for $CaCl_2$ (Fig. 5). Several observations support this conclusion. First, though K was determined for most salts with use of only two values of T_m, a straight line passed through these points intersects the ordinate in each case at essentially the same value of T_m^0 (20.2 ± 0.7°). Furthermore, Bello *et al.* (1956) have shown for several salts that the melting point of 5% gelatin gels also varies directly with salt concentration (see Discussion).

Inspection of Figure 7 shows that the specific rotation at the low temperature end of melting curves obtained at high salt concentrations (with electrolytes characterized by a large negative K) are substantially lower than those measured at lower concentrations of the same salt. (Compare with Figure 4, for $CaCl_2$.) In order to demonstrate for the general case that the *equilibrium* amount of helix formed is independent of m, $\Delta[\alpha]$ (as defined for Figure 2) was measured for several gelatin-electrolyte systems after several weeks at 5°. The results were plotted as in Figure 2. In all cases, after long periods at 5°, $\Delta[\alpha]$ approached a constant value for all but the very highest concentrations of salt.

The relationship between the kinetic parameter k_0 and the equilibrium parameter K is shown in Figure 8 for a number of salts. This plot brings out the fact that various salts affect these two parameters to different extents: *e.g.*, in the $(NH_4)_2SO_4$ - $(CH_3)_4NCl$ - KCH_3COO region, k_0 remains very close to zero whereas K varies over a considerable range. On the other hand, at the $CaCl_2$-KSCN end of the plot, k_0 (proportionally) changes more rapidly than K.

The data of Table I (in conjunction with Figure 8) suggest that substituting methyl groups for hydrogens on the ammonium ion results in the conversion of this ion from a weak melting-point reducer to an additive which markedly *raises* T_m. Hence it seemed of interest to determine what effect further increases in the size of the ammonium substituents might have on K and k_0. Table II shows the effects of comparable concentrations of tetramethyl-, tetraethyl-, tetrapropyl-, and tetrabutylammonium bromide on the initial rate of collagen-fold formation and on T_m. It is clear that the effects do not continue in the direction suggested by the NH_4^+ to $(CH_3)_4N^+$ progression. Rather $(C_2H_5)_4N^+$ depresses T_m and $(d[\alpha]/dt)_0$ somewhat, and $(C_3H_7)_4N^+$ and $(C_4H_9)_4N^+$ inhibit collagen-fold formation altogether at temperatures above 7°. A similar effect of increasing the aliphatic chain-length of organic acids on the melting point of concentrated gelatin gels has been described by Bello *et al.* (1956).

Finally, we have compared the results obtained with ichthyocol gelatin with data obtained with a gelatin of different amino acid composition and properties. Table III compares the values of K, k_0, and r_0 obtained with the ichthyocol gelatin–$CaCl_2$ and the calfskin gelatin–$CaCl_2$ systems. As previously observed (Harrington and von Hippel, 1961b) and expected on the basis of its increased content of imino acids, calfskin gelatin was found to have an r_0 several times greater than that for ichthyocol. K for calfskin is also somewhat elevated, but k_0 for both systems is almost identical.

DISCUSSION

In this study we have examined the effect of neutral salts on both kinetic and equilibrium properties of the collagen-type helix at very low gelatin concentrations. The results have been expressed in terms of the parameter k_0, which may be used as a comparative measure of the effect of various salts on the kinetics of formation of the collagen-fold, and K, which serves to quantitate the effect of various electrolytes on the stability of the equilibrium structure. Previous investigations of salt effects on gelatin have generally been carried out at rather high concentrations of gela-

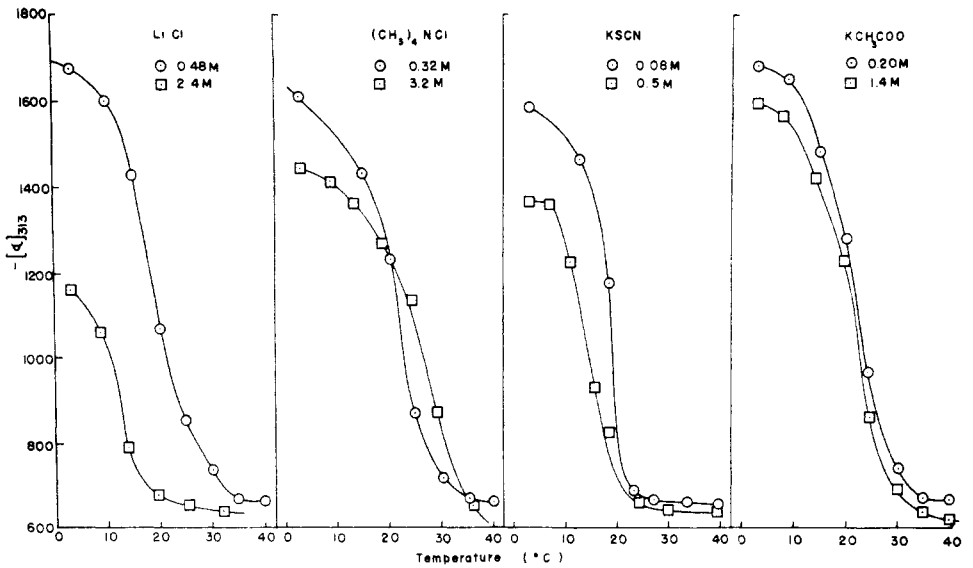

FIG. 7.—Specific rotation of samples of ichthyocol gelatin in various salts as a function of temperature, after 24 hours at 5° *p*H 7; protein conc. ≃ 1.2 mg/ml.

tin and have focussed only on measurements relating to the equilibrium situation. A comparison of our results with some of this earlier work is of particular interest in view of these concentration differences (see below).

Several groups, particularly Carpenter (1938) and his associates and Katz and Wienhoven (1933), have used optical rotatory methods to study the effect of neutral salts on gelatin. These workers used calfskin gelatin at concentrations well above the gelation limit, and attempted to quantitate the salt effects by measuring the specific rotation of gels held at a constant low temperature until the rotation had essentially stopped changing. The rotations observed under these conditions at various salt concentrations were compared with values obtained on the same samples at a temperature *above* the melting point,

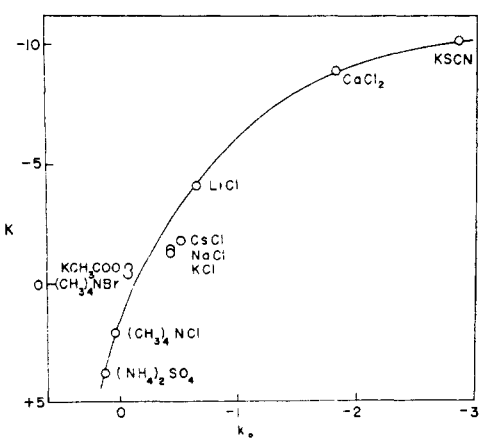

FIG. 8.—Relation between kinetic parameter, k_0, and equilibrium parameter, K, for various salts. k_0 and K defined by equations (1) and (2), see text. k_0 measured at ~3°.

and a quantity $\Delta[\alpha]_7$ (defined as the difference between the low and the high temperature rotation at a given salt concentration) was calculated for a number of electrolytes at various concentrations. By use of such data, the molar effectiveness of various ions in reducing $\Delta[\alpha]$ can be qualitatively compared; studies carried out using a series of salts with one ion in common made it possible to rank anions and cations separately. In order of *decreasing* molar effectiveness in reducing $\Delta[\alpha]$, Carpenter and co-workers obtained the following series for monovalent anions: $CNS^- > I^- > ClO_3^- > NO_3^- > Br^- > Cl^- \simeq CH_3COO^-$. The monovalent cations were ranked as follows: $Li^+ > Na^+ > Cs^+ > Rb^+ > K^+$,

TABLE II

EFFECT OF VARIOUS SUBSTITUTED AMMONIUM IONS ON THE FORMATION AND STABILITY OF THE COLLAGEN-FOLD[a]

Salt	$\left(\frac{d[\alpha]_{313}}{dt}\right)_0$	T_m
$(CH_3)_4NBr$	−8.2°/min (1 M)	18.4°C (2 M)
$(C_2H_5)_4NBr$	−2.8 (1 M)	15.4 (2 M)
$(C_3H_7)_4NBr$	0 (1 M)	—
$(C_4H_9)_4NBr$	0 (1 M)	—

[a] Ichthyocol gelatin; protein conc. ≃1.2 mg/ml. Initial rates measured at 7.0°.

TABLE III

COMPARISON OF THE EFFECT OF $CaCl_2$ ON ICHTHYOCOL AND CALFSKIN GELATINS

	Ichthyocol	Calfskin
r_0	−35°/min (3.8°)	−160°/min (2.0°)
k_0	−1.8	−1.7
K	−8.8	−12.8

though these differed in effectiveness much less than did the anions. Katz and Wienhoven, using a similar criterion, arrived at the same anion series. In addition, these workers found that $SO_4^=$ and F^- *increased* the specific rotation in the cold over the value found without added salt. Comparison of these series with either the K or the k_0 values listed in Table I shows general qualitative agreement in terms of the *order* of effectiveness of the various ions.

More quantitative comparison can be made with the data obtained on high isoelectric point pigskin gelatin by Bello *et al.* (1956). These investigators cooled 5% gelatin solutions containing various salts, and measured the melting temperature of the resulting gels as the temperature was progressively increased at a rate of 5° per hour. Neoprene balls were inserted in the solid gel and the melting point (m.p.) was defined as the bath temperature at which a ball reached the bottom of a given tube. This criterion was found to be very reproducible, and, although it is not clear to which point on an equilibrium melting curve this temperature corresponds, *changes* in m.p. can be compared directly with changes in T_m. Bello and co-workers showed, as pointed out above, that the melting point of the gel varied linearly with molarity of added salt. Thus values of K (defined as the change in melting temperature per mole of added salt) may be calculated from their data and compared with the results obtained in dilute solution in this study. Such a comparison is made in Table IV. Clearly the values of K obtained for the various gelatins with each salt are similar. In fact, those for pigskin (5% gel) and calfskin (~1 mg/ml solution) are virtually identical.[1]

The finding that the change in melting point per mole of added salt is independent of gelatin concentration has several important consequences. First, it is well known that the stability of both collagen fibers and gelatin gels (as measured by the temperature of the collagen-fold $\rightleftarrows$ gelatin transition) may be viewed as the sum of a concentration-dependent and a concentration-independent component (Ferry and Eldridge, 1949; Boedtker and Doty, 1956; Flory and Garrett, 1958; Harrington and von Hippel, 1961b). In these terms, it may now be seen that neutral salts must alter the melting temperature via an *intra*-molecular mechanism, acting directly on the structure of the collagen-type helix lying *between* cross-links rather than attacking the interchain cross-links themselves.

With respect to the actual *mechanism* of this effect, there appear to be three general types of possibilities: (1) a general electrostatic or "ionic strength" effect; (2) direct ion-binding to specific chemical groupings on the gelatin chain; or (3) specific effects on solvent associated with the gelatin chains.

[1] It is interesting to note, in this connection, that pigskin and calfskin gelatins have almost the same amino acid composition (Eastoe, 1955; Piez and Gross, 1960), both containing substantially more proline and hydroxyproline than does ichthyocol.

TABLE IV

COMPARISON OF THE MOLAR EFFECTIVENESS OF VARIOUS SALTS IN ALTERING THE COLLAGEN-FOLD $\rightleftarrows$ GELATIN TRANSITION TEMPERATURE[a]

Salt	$K_{(\text{pigskin})}$	$K_{(\text{ichthyocol})}$	$K_{(\text{calfskin})}$
$CaCl_2$	−14.2	−8.8	−12.8
NaSCN	−14.4	−10.2	(−14.8)
LiCl	−5.0	−4.1	(−6.0)
NaCl	−2.4	−1.8	(−2.6)

[a] K measured in °C/mole of added salt. Pigskin data (5% gels) from Bello *et al.* (1956); ichthyocol and calfskin data (~1 mg/ml solutions), this study. K for the ichthyocol-NaSCN system was calculated by adding −0.2 to the value measured for ichthyocol-KSCN (based on the difference between K for the NaCl and the KCl systems [Table I] and the demonstration by Bello *et al.* that the effects of the anion and cation of an electrolyte system are additive). The calfskin-$CaCl_2$ value was experimentally determined. The rest of the calfskin-salt data were calculated from the corresponding ichthyocol data by multiplying by the ratio of $K_{(\text{ichthyocol-CaCl}_2)}$ to $K_{(\text{calfskin-CaCl}_2)}$.

Mechanisms of type 1 can be dismissed immediately, on the grounds that identical concentrations of salts of the same valence-type have such widely different effects (*e.g.*, contrast LiCl and $(CH_3)_4NCl$ or $BaCl_2$ and $(NH_4)_2SO_4$).

Alterations in the stability of the collagen-fold as a consequence of specific ion-binding can also be ruled out, largely on the basis of studies by Bello *et al.* (1956, 1962b) on the effect on the melting temperature of specifically blocking various charged groups. These workers demonstrated that neither preventing anion binding at amino, guanidino, or hydroxyl groups, nor blocking cation binding at carboxyl or hydroxyl groups, altered the effect of ions on the melting point of the gels. Nevertheless, they felt it very likely that ion-binding was involved in some form and concluded (essentially by default) that ion complexes with the peptide groups must be responsible for the observed salt effects.

However, a comparison of our data in dilute solution with the results of Bello and co-workers on gels can be used to absolve virtually *any* type of ion-binding of responsibility for the melting-point effects. Describing the direct binding of a salt to a specific site on the gelatin chain in the usual way, we may write:

$$\frac{[SG]}{[S][G]} = K_{eq} \qquad (3)$$

where $[S]$ = concentration (activity) of free salt, $[G]$ = concentration of free binding sites, $[SG]$ = concentration of *filled* binding sites, and K_{eq} = the equilibrium constant for the system. Equating G with the peptide bond, as suggested by Bello *et al.*, we calculate $[G]$ for the 5% gelatin gels used by these workers to be approximately

0.5 M. On the other hand, in our dilute solution studies the concentration of peptide groups ([G]) was only about 0.01 M. Assigning either a large or a small value to K_{eq}, it can easily be shown that one cannot account for either the linearity of the relation between T_m and m (equation 2) or the lack of dependence of K on gelatin concentration (Table IV), especially at hypothetical binding site concentrations of the same order of magnitude as the concentration of added salt, by any mechanism which requires that ions alter the stability of the helix by *direct* binding to peptide groups. The same argument applies if one equates G with any other specific group on the polypeptide chain. In fact, it is clear that the neutral salt effect cannot be attributed to any mechanism which depends directly on gelatin concentration.[2]

Thus we are left with the third type of mechanism suggested above: namely that stabilization of the collagen-fold somehow involves specific interactions between the gelatin chains and the solvent (water), and that ions affect the helix indirectly by interacting with such structurally involved water molecules.

It is well known that many properties of electrolytic solutions can be rationalized in terms of specific interactions between ions and solvent. In particular, it is clear that ions distort the lattice structure characteristic of pure water by competitively reorganizing neighboring water molecules into new structures. The nature and extent of this reorganization vary greatly from one ion to another; *e.g.*, see Frank and Evans (1945). Since the average center-to-center separation of ions in an 0.01 M uni-univalent electrolyte solution is only about 44 A (Robinson and Stokes, 1959), it is clear that even at relatively low concentrations of salt essentially every water molecule lies within the sphere of influence of at least one ion. Thus if specific interactions between gelatin and water are involved in forming and stabilizing the collagen-fold, one would expect that these interactions might be modified in the presence of various salts. Depending on the nature of the interaction between specific ions and water, some salts might *increase* the stability of the collagen-fold by moderating the competitive effect of the undistorted water lattice itself, while other salts might *reduce* the stability of the helix by competing directly for the structural water.

As a first approximation, we may determine whether there is a direct correlation between the effectiveness of various ions in reducing the activity of water and their effectiveness in lowering the stability of the collagen-fold. The activity of water in various concentrations of the salts listed in Table I was calculated from the tables of osmotic coefficients provided by Robinson and Stokes (1959). These calculations showed, especially for anions, that there is little or no qualitative or quantitative correlation between the effect of an electrolyte on the activity of water and its effect on the stability of the collagen-fold. This is, perhaps, not surprising, if the effect on the collagen-fold is viewed as the resultant of a complex three-component interaction (between gelatin and water, salt and water, and water and water) to which the contributions of all components must be taken into account. In order to approach this problem more directly, we must consider in detail how water molecules might actually be involved in the formation and stabilization of the collagen-fold.

It has previously been suggested, on the basis of experiments in D_2O and on stereochemical grounds (von Hippel and Harrington, 1960; Harrington and von Hippel, 1961b), that water molecules may participate in the formation and stabilization of the collagen-type helix by forming double-ended hydrogen-bonded bridges between adjacent carbonyl oxygens along individual gelatin chains, and thus help to "lock" the chain into the poly-L-proline II configuration characteristic of the collagen-fold. It was further proposed that the gelatin $\rightleftarrows$ collagen-fold phase transition (proceeding toward the ordered form) is nucleated by the "locking" of pyrrolidine-rich regions of the gelatin chain into the poly-L-proline II structure. This "locking" was assumed to take place both because isomerization of the pyrrolidine rings about the polypeptide chain becomes progressively more difficult as the temperature is lowered, and simultaneously because the proposed intramolecular hydrogen-bonded water bridges between adjacent carboxyl groups become progressively more stable. It was then suggested that the "locked" regions serve as active crystallization nuclei for a linear phase transition which propagates along the chain through the formation of more water bridges as the structure progressively twists into the poly-L-proline II configuration. Thus, in terms of this model, though nucleation and growth were considered to be two distinct processes, water as a structural element was im-

[2] The effect of divalent copper and nickel on gelatin is probably an exception to this statement. Very low concentrations of these ions at high pH completely inhibit gelation (Bello and Vinograd, 1958; Bello *et al.*, 1961b) and mutarotation (von Hippel and Wong; cited in Harrington and von Hippel, 1961a), presumably by forming a biuret-type complex across the peptide bond and thus inhibiting the development of the poly-L-proline II-type helix by restraining adjacent residues in sterically unfavorable configurations. These effects can be completely reversed by lowering the pH, as one might expect if a biuret-type complex is involved. In addition to this pH sensitivity, the effect of Cu^{++} and Ni^{++} also differs markedly from that of other salts in that T_m is definitely *not* linearly related to Cu^{++} (or Ni^{++}) concentration (see Fig. 4, Bello *et al.*, 1961b), and in the extremely low ion concentrations required to suppress gelation completely, even at 0°. All these factors suggest that copper and nickel ions at high pH affect gelation via a rather unique binding mechanism which differs markedly from the mechanism of action of most of the neutral salts.

plicated in both.[3] The present data on ion effects can now be reconsidered in the light of this model.

As has been pointed out above, the ion effects which we have observed can be described with two parameters, k_0 and K. In terms of the above model, k_0 can be viewed as a measure of the effects of various salts on the nucleation phenomenon, and K can be considered to be a measure of the stability of internuclear regions. Since the gelatin $\rightleftarrows$ collagen-fold phase transition is heterogeneously nucleated (*i.e.*, regions in which nuclei can develop presumably differ in amino acid composition from prospective internuclear regions) the effect of salts on the water molecules assumed to be involved in stabilizing the active nuclei may not be identical to the effect on water in internuclear regions. This suggestion is in accord with the results presented in Figure 8, in which k_0 is plotted against K for several salt systems. Figure 8 shows that certain salts affect K markedly while influencing k_0 very little, whereas other salts have a proportionately greater effect on k_0 than on K.

Certain features of the nucleation phenomenon may be deduced from the form of the dependence of initial rate on time and salt concentration. First, in no case is a lag-phase observed, suggesting that all nuclei which are going to exceed critical size and thus propagate the transition begin to grow at time zero (directly after quenching). Furthermore, the exponential dependence on the molarity of salt [equation (1)] might be interpreted by assuming that electrolytes affect the initial rate of helix formation by altering the *number* of active growth nuclei as follows:

$$r/r_0 = N^*/N_0 = e^{k_0 m} \qquad (4)$$

where N^* = the number of active growth nuclei at temperature T and concentration of salt m, and N_0 = the number of active growth nuclei at temperature T and $m = 0$. In this case we assume that all active growth nuclei propagate at an initially constant rate. An alternative interpretation might be that the number of active nuclei remains unchanged, but that increasing concentrations of salt alter the average initial *rate* in accord with equation (4).

Both the form of equation (4) and our conclusion that ions affect the collagen-fold through a competitive reorganization of the solvent are very reminiscent of the "salting-out" of proteins (and other solutes) by high concentrations of electrolytes. This phenomenon, which has generally been interpreted in terms of a competitive removal of water molecules from their solvent role (with respect to the protein or other solute) through preferential interaction with the ions (Debye, 1927; Frank and Evans, 1945; Long and McDevit, 1952; Edsall and Wyman, 1958) is generally described by an equation of the following type:

$$S^*/S_0 = e^{k_s m} \qquad (5)$$

where S^* = the solubility of the protein at salt concentration m, S_0 = the hypothetical extrapolated solubility at $m = 0$, and k_s = the "salting-out" constant which is characteristic of a given protein-salt system. The similarity between equations (4) and (5) is striking. The "salting-out" phenomenon and the effect of ions on the collagen-fold have several other features in common:

(1) k_s for protein-salt systems varies between approximately -0.8 and -3.0 (see Edsall and Wyman, 1952). The range of k_0 is similar (Table I).

(2) The salting-out constant for a given protein-salt system is independent of *p*H (and thus of protein charge); (*e.g.*, see Green, 1931). Figures 1 and 2 suggest that this is also true for k_0. In addition, various workers have shown that the melting point (and therefore K) of a gelatin gel at a given salt concentration is also essentially independent of *p*H (Pleass, 1930; Gordon, 1948; Bello *et al.*, 1956, 1962a).

(3) In a homologous series, the addition of aliphatic carbons to a hydrocarbon chain causes a progressive decrease in the dielectric constant of an aqueous solution. This effect is presumably due to a progressive rearrangement of the solvent about those nonpolar molecules (Frank and Evans, 1945). This change is associated with an increased tendency toward "salting-out" (Edsall and Wyman, 1945) and is also accompanied by a reduction in the rate of formation and in the stability of the collagen-fold (see Table II; also Bello *et al.*, 1956, Fig. 1).

On the other hand, it should be pointed out that both of the salts which precipitated (or "salted-out") gelatin in this study (KCH_3COO and $(NH_4)_2SO_4$) are characterized by very small values of k_0. Therefore, though both phenomena appear to be based on a competitive reorganization of the solvent by the ions, the details of the interaction doubtless differ somewhat.

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Added in Proof

Since this paper has been in press, a number of colleagues (particularly Dr. J. Belló of the Roswell Park Memorial Institute) have pointed out to us that the argument in the Discussion, which is intended to demonstrate that direct binding of

[3] In experiments currently in progress, the participation of bound (and thus less exchangeable) water in the formation of the collagen-fold is being directly examined by means of a hydrogen-tritium exchange technique. A preliminary account of this method and some of its applications has been presented (Englander, 1962).

ions to the gelatin chain cannot be responsible for the neutral salt effect, is given so briefly as to be misleading. Thus, in order to avoid possible misconceptions concerning this rather important point, the argument is here presented in somewhat more detail.

If specific binding of ions to chemical groups along the gelatin chain were responsible for the effects observed, presumably by altering the conformational possibilities open to a segment of the chain and thus locally inhibiting formation of the poly-L-proline II-type helix, one could conceive of four extreme situations in terms of equation (3).

1. K_{eq} *large and G large:* *i.e.*, most of the added salt is bound and there is at least one "effective" binding site for every three to four residues. G here is defined as the total number of "effective" sites and "effective" binding implies an event which inactivates a segment of the chain as a potential helix forming region. (It is clear that ion-binding to gelatin does take place; the question at issue is whether this has any effect on the formation of the collagen-fold.) This possibility can be ruled out by the observed lack of dependence of K (the slope of the T_m *vs.* m plot) on gelatin concentration, since if K_{eq} is large, one should be able to saturate the effective binding sites with only a small excess of salt. Therefore, the salt concentration at which helix formation is completely suppressed should vary directly with gelatin concentration.

2. K_{eq} *large and G small:* *i.e.*, the equilibrium constant favors ion-binding and there are very few effective binding sites per chain, which when filled each inactivate large segments of the chain in terms of helix-forming potential. This is mechanistically rather unlikely since Yaron and Berger (*Bull. Res. Council Israel 10A*, 46, 1961) have shown for poly-L-proline and we for gelatin (von Hippel and Wong, in preparation) that relatively short chain segments can go into the poly-L-proline II helix. However, this case can also be ruled out formally on the same basis as case (1), since here at low gelatin concentrations even trace amounts of salt would be expected to suppress helix formation completely.

3. K_{eq} *small and G large:* If K_{eq} is small, the lack of dependence of the effect on gelatin concentration cannot, of course, be used as an argument since it can easily be shown that even at fairly large salt concentrations most of the binding sites remain free and the ratio $[SG]/[G]$ (eq. 3) is small and essentially independent of gelatin concentration. On the other hand, for many salts (see Table I) suppression of helix formation *does* go to completion at concentrations of salt of the same order of magnitude as the assumed concentration of potential binding sites (at least for the 5% gels) which is impossible if K_{eq} is small and G large.

4. K_{eq} *small and G small:* This possibility seems mechanistically unlikely for the same reasons as case (2), but can also be formally ruled out by the observation that at a given temperature complete suppression of helix formation *can* be brought about, implying in terms of this model that sufficient salt *can* be added to fill essentially all of the binding sites. Hence by adding salt one can completely titrate the "effective" sites, and therefore in terms of equation (3) one would expect the effect of successive aliquots on the stability of the helix (measured as a change in T_m) to follow a simple adsorption isotherm, the effect per aliquot decreasing progressively with increasing concentration of salt. However, the demonstrated linearity of T_m with respect to added salt indicates that this is not the case.

To sum up, direct binding of ions to gelatin, no matter what value of K_{eq} or G is assumed, is incompatible with at least one of the following three experimental facts: (*a*) The effect is independent of gelatin concentration; (*b*) T_m is a linear function of molarity of added salt; and (*c*) complete suppression of helix formation can be achieved with a variety of salts at moderate concentrations. Therefore ion-binding, *per se*, cannot serve as an explanation of the observed neutral salt effects.

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Polymerization Reactions Catalyzed by Intracellular Proteinases. III. Action of Cathepsin C on a Tetrapeptide Amide*

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When cathepsin C acts at *p*H 7.5 on glycyl-L-tyrosylglycyl-L-tyrosinamide labeled with C^{14} in one of the two glycine residues, the labeling of the resulting polymeric peptide amide indicates that both dipeptide units are incorporated, but only after prior cleavage of the interior tyrosylglycyl bond. The data are in accord with the hypothesis that the tetrapeptide amide is an intermediate in the enzymic polymerization of glycyl-L-tyrosinamide only when the tetrapeptide amide is formed and held by the enzyme.

In previous communications from this laboratory, it was reported that at *p*H 7.5, cathepsin C converts glycyl-L-tyrosinamide to a decapeptide amide (Fruton *et al.*, 1953), and that during this polymerization reaction glycyl-L-tyrosylglycyl-L-tyrosinamide appears in the incubation mixture (Würz *et al.*, 1962). Experiments designed to determine the role of the tetrapeptide amide in polymer formation indicated that this compound, when added to the incubation mixture, did not serve as a "primer" in the polymerization of glycyl-L-tyrosinamide. When synthetic tetrapeptide amide was present in an incubation mixture containing C^{14}-labeled glycyl-L-tyrosinamide, the radioactivity of the resulting polymer was reduced, as compared with a control experiment in the absence of tetrapeptide amide, indicating that the unlabeled tetrapeptide amide had contributed glycyltyrosyl units to the formation of the polymer. Furthermore, cathepsin C was shown to act on glycyl-L-tyrosylglycyl-L-tyrosinamide at *p*H 7.5 with the formation of glycyl-L-tyrosine and of polymer, and with slow evolution of ammonia, indicating a primary attack of the enzyme at the interior tyrosylglycyl peptide bond. The available data led to the suggestion that in the polymerization of glycyl-L-tyrosinamide by cathepsin C, the tetrapeptide amide only serves as an intermediate when it remains bound to the catalytic region of the enzyme; if released from the site of its formation, or if added to the incubation mixture, the tetrapeptide amide appears to be cleaved to dipeptide units (possibly in the form of glycyltyrosyl-enzyme) before re-utilization for polymer formation.

In the present communication, further data are presented in support of the conclusion that free glycyl-L-tyrosylglycyl-L-tyrosinamide, when acted upon by cathepsin C, is cleaved before utilization for polymer formation.

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