The Decomposition of Soluble Collagen by γ -Irradiation

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1. The effect of γ -irradiation in the range 1 krad–10 Mrads on freeze-dried acid-soluble collagen was studied. 2. The specific-rotation and reduced-viscosity recoveries after heating and cooling of the irradiated collagen in solution showed a high degree of dependence on irradiation dose, with reduced viscosity showing significantly less recovery than specific rotation on increasing the irradiation dose. 3. The dependence of reduced viscosity on concentration was greatly decreased with increased doses of γ -irradiation. 4. The melting temperature measured by optical rotation also decreased as the irradiation dose was increased, and at low doses was distinctly biphasic. 5. Physical properties showed that the action of γ -irradiation up to 10 Mrads occurred in two distinct phases, with the early changes being extremely sensitive to irradiation dose. 6. The action of the γ -irradiation is discussed in terms of the structure of tropocollagen.

A previous study of the effect of γ -irradiation on soluble collagen over the range 1.3–10 Mrads (Davidson & Cooper, 1968) has been expanded in the intermediate dose range (1.0–500 krad) to examine the progressive structural changes occurring in the tropocollagen molecule in the region of relatively restricted damage encountered at low irradiation doses. As in the previous study, irradiation treatments were carried out on the freeze-dried collagen *in vacuo* to minimize complications arising from the 'indirect effect', involving attenuation of the available irradiation intensity with accompanying production of free radicals in the solvent during exposure of collagen solutions. It has been reported, however, that free radicals can also be formed during the irradiation of dry proteins (e.g. Copeland, Sanner & Pihl, 1968), so that a possible contribution due to the presence of free radicals cannot be disregarded entirely in the irradiation of dry collagen.

The results of previous physicochemical evaluation in terms of mutarotation and reduced-viscosity recoveries after heating and cooling of γ -irradiated collagen in solution (Davidson & Cooper, 1968) showed a high degree of dependence on irradiation dose, consistent with the principal mechanisms of depolymerization and fragmentation by fission of peptide bonds proposed. The present study details the effects of γ -irradiation on the physicochemical characteristics of acid-soluble collagen with particular reference to changes occurring at intermediate doses not reported previously.

MATERIALS AND METHODS

Preparation of acid-soluble collagen. Acid-soluble collagen was prepared from calf skin by the methods described before (Cooper & Davidson, 1965; Davidson & Cooper, 1968). Neutral-salt-soluble collagen was extracted from the skin before preparation of the acid-soluble collagen, which was finally dialysed against 0.15M-acetic acid before being freeze-dried.

γ -Irradiation. Freeze-dried samples of the acid-soluble collagen were dried under vacuum over P_2O_5 for 24 hr., sealed under vacuum into glass tubes and irradiated at 22° from a 300–400 c ^{60}Co source by the South African Atomic Energy Board. The rate of irradiation was 1.2 krad/min.

Mutarotation at 15°. The procedure for following collagen-fold re-formation at 15° after heat-denaturation (for 15 min. at 45°) of collagen in 0.15M-potassium acetate buffer, pH 4.8, by using viscometry and polarimetry has been described by Davidson & Cooper (1967). Viscosities were measured in Canon-Fenske viscometers (size 50 and 100, B.S. 188), and optical rotations in 10 cm. water-jacketed tubes by a Perkin-Elmer model 141 polarimeter operating at 365 nm.

Melting curves. Melting curves were obtained by polarimetry by the '30 min. method' (Hippel & Wong, 1963a).

RESULTS

Mutarotation. To study the effect of γ -irradiation in the range 1 krad–10 Mrads, solutions of irradiated and non-irradiated acid-soluble collagen (1.2–1.4 mg./ml.) were denatured at 45° for 15 min. and helix re-formation was monitored by measurements of optical rotation at 15°. The results for doses in the

range 1.3–10 Mrads have been illustrated previously (Davidson & Cooper, 1968), and the remaining results are given in Fig. 1. This information is summarized in Table 1, which shows that both the initial rates of mutarotation and the actual recoveries after 48 hr. decrease as the irradiation dose increases; a similar progressive decrease with irradiation dose was also observed in the specific rotation of the collagen solutions containing irradiated collagen before thermal denaturation (Table 1). Thus the mutarotation recovery, being the specific-rotation value attained after 48 hr. at 15° expressed as a percentage of the specific rotation of the corresponding original solution containing irradiated material with no history of heating, is remarkably constant at about 80% for all the doses of γ -irradiation (Table 1), whereas the mutarotation recovery expressed as a percentage of the specific rotation of the non-irradiated native

acid-soluble collagen decreased from 81% to 62% (Table 1).

The relationships between the amount of irradiation and the specific rotation at 15° of the solutions with no thermal history above this temperature, and the mutarotation values after heating, attained after 48 hr. at 15°, are shown in Fig. 2. These results show a rapid decrease in the specific-rotation and mutarotation values with the initial relatively small doses of γ -irradiation, up to about 100 krad. As the dose is increased a steady decrease in these values occurs at a lower rate.

Viscosity recovery. A parallel study was made of the regain in reduced viscosity at 15° after thermal denaturation. Increased doses of γ -irradiation caused a decrease in the initial rate of viscosity recovery (Fig. 3 and Davidson & Cooper, 1968) and in the reduced viscosity after 48 hr. at 15° (Table 2).

The recovery in reduced viscosity expressed as a percentage of the reduced viscosity of the corresponding collagen sample with no thermal history (Table 2) is not as constant as a similar relationship measured by specific rotation (Table 1), and has a much lower value (about 16%). The same recovery values expressed as a percentage of the reduced viscosity of the original non-irradiated collagen ($[\eta]_{\text{red}}^{15} = 41.2$) decrease markedly from 16% to 2.5% (Table 2). Therefore the recovery in reduced viscosity on cooling thermally denatured solutions is very much lower than the recovery in specific rotation. Further, the irradiation has a bigger effect on viscosity than on optical rotation of unheated solutions. Thus after 10 Mrads the specific rotation falls to 73% of the specific rotation of non-irradiated collagen (Table 1), whereas the reduced viscosity falls to 12% of the reduced viscosity of non-irradiated collagen (Table 2).

The relationships between the irradiation dose

Fig. 1. Specific rotation ($[\alpha]_{365}^{15}$) of γ -irradiated acid-soluble collagen as a function of time. Solutions (1.2–1.4 mg./ml.) were thermally denatured at 45° for 15 min. and then kept at 15°. ○, Not irradiated; △, 1 krad; □, 10 krad; ●, 100 krad; ▲, 500 krad.

Table 1. Optical rotation of acid-soluble collagen after γ -irradiation

Irradiation dose	Specific rotation at 15° of collagen after irradiation ($[\alpha]_{365}^{15}$)	Specific rotation of irradiated collagen (% of that for native collagen)	Specific rotation after thermal denaturation and cooling to 15° for 48 hr. ($[\alpha]_{365}^{15}$) (mutarotation recovery)	Mutarotation recovery (% of specific rotation of native collagen)	Mutarotation recovery (% of specific rotation of corresponding unheated collagen)
0	-1261°	100	-1025°	81	81
1 krad	-1228	98	-991	79	81
10 krad	-1215	96	-956	76	79
100 krad	-1191	95	-944	75	79
500 krad	-1192	96	-926	73	78
1.3 Mrads	-1199	95	-950	75	79
2.6 Mrads	-1145	91	-927	73	81
5 Mrads	-1046	83	-827	66	79
10 Mrads	-914	73	-783	62	86

Fig. 2. Specific rotation ($[\alpha]_{365}^{15}$) of γ -irradiated acid-soluble collagen as a function of irradiation dose. Solutions (1.2–1.4 mg./ml.) were measured before heating (○), and after heating at 45° for 15 min. and keeping at 15° for 48 hr. (●).

Fig. 3. Reduced viscosity ($[\eta]_{red.}^{15}$) of γ -irradiated acid-soluble collagen as a function of time. Solutions (1.2–1.4 mg./ml.) were thermally denatured at 45° for 15 min. and then kept at 15°. ○, Not irradiated; Δ, 1 krad; □, 10 krad; ●, 100 krad; ▲, 500 krad.

and the reduced viscosity ($[\eta]_{red.}^{15}$, corrected to 1.2 mg./ml.; see Fig. 5) for unheated solutions and the viscosity recovery after 48 hr. at 15° after thermal denaturation respectively are shown in Fig. 4. These relationships are comparable with similar studies carried out by specific-rotation measurements (Fig. 2), except that the reduced viscosity of the unheated samples falls very rapidly with initial low doses.

The dependence of reduced viscosity on concentration at each irradiation dose is shown in Fig. 5. It is evident that, as the amount of irradiation is increased, the concentration-dependence of the reduced viscosity decreases very significantly. Since it is difficult to determine the reduced viscosity at zero concentration (i.e. the intrinsic viscosity or limiting viscosity number, $[\eta]$) from extrapolation of these curves, the inherent viscosity ($[\ln(\eta/\eta_0)]/c$) was plotted against concentration (Yang, 1961).

Fig. 4. Reduced viscosity ($[\eta]_{red.}^{15}$) of γ -irradiated acid-soluble collagen as a function of irradiation dose. Solutions (1.2–1.4 mg./ml.) were measured before heating (○), and after heating at 45° for 15 min. and keeping at 15° for 48 hr. (●).

Table 2. Viscosity of acid-soluble collagen after γ -irradiation

Irradiation dose	Reduced viscosity at 15° of collagen after irradiation ($[\eta]_{red.}^{15}$ at 0.12 g./100 ml.) (dl./g.)	Reduced viscosity of irradiated collagen (% of that for native collagen)	Viscosity recovery after thermal denaturation and cooling to 15° for 48 hr. ($[\eta]_{red.}^{15}$) (dl./g.)	Viscosity recovery (% of reduced viscosity of native collagen)	Viscosity recovery (% of reduced viscosity of corresponding unheated collagen)	Limiting viscosity number (lim. $[\eta]_{red.}^{15}$ $c \rightarrow 0$) (dl./g.)
0	41.2	100	6.68	16.0	16	16.3
1 krad	28.0	68	5.72	14.0	20	13.5
10 krad	28.2	69	4.92	12.0	17	13.1
100 krad	27.3	66	4.00	9.7	15	12.1
500 krad	24.2	59	3.61	8.8	15	11.4
1.3 Mrads	32.2	78	4.08	9.9	13	12.7
2.6 Mrads	22.3	54	3.59	8.7	16	11.0
5 Mrads	12.0	29	1.65	4.0	14	6.6
10 Mrads	4.8	12	1.04	2.5	22	2.9

Fig. 5. Reduced viscosity ($[\eta]_{red.}^{15}$) of γ -irradiated acid-soluble collagen as a function of concentration (mg./ml.). $\circ$, Not irradiated; $\triangle$, 1 krad; $\square$, 10 krad; $\bullet$, 100 krad; $\blacktriangle$, 500 krad; $\blacksquare$, 1.3 Mrads; $\odot$, 2.6 Mrads; $\blacktriangle$, 5 Mrads; $\blacksquare$, 10 Mrads.

Fig. 6. Limiting viscosity number ($\lim_{c \rightarrow 0} [\eta]_{red.}^{15}$) of γ -irradiated acid-soluble collagen as a function of irradiation dose.

This gave a linear relationship and accurate extrapolation to zero concentration (Table 2). The relationship between limiting viscosity number and irradiation dose is recorded in Fig. 6. Once again these data show an initial rapid decomposition at doses below 100 krad.

Melting curves. The melting curves for non-irradiated and irradiated acid-soluble collagen are given in Fig. 7, from which values can be obtained for T_m , the temperature at the mid-point of the transition (melting point), $\Delta[\alpha]_T$, the difference between the specific rotation at the high and low

Fig. 7. Specific rotation ($[\alpha]_{365}^{15}$) of γ -irradiated acid-soluble collagen as a function of temperature. $\circ$, Not irradiated; $\triangle$, 1 krad; $\square$, 10 krad; $\bullet$, 100 krad; $\blacktriangle$, 500 krad; $\blacksquare$, 1.3 Mrads; $\odot$, 2.6 Mrads; $\blacktriangle$, 5 Mrads; $\blacksquare$, 10 Mrads.

Fig. 8. Melting temperature of γ -irradiated acid-soluble collagen as a function of irradiation dose.

Table 3. *Melting point of acid-soluble collagen after γ -irradiation*

Irradiation dose	Melting point T_m		$(\Delta[\alpha]_{365})_T$	ΔT
	Low	High		
0	—	38.6°	-828°	1.2°
1 krad	34.4°	38.6	-804	1.6
10 krad	34.6	38.8	-786	2.0
100 krad	33.8	—	-770	2.7
500 krad	33.4	—	-765	2.9
1.3 Mrads	32.8	—	-790	3.8
2.6 Mrads	31.0	—	-736	3.2
5 Mrads	30.6	—	-662	4.6
10 Mrads	29.2	—	-563	7.0

temperature ends of the transition, and ΔT , the difference in temperature between the points at which the helix-coil transition is one-quarter and three-quarters complete (Hippel & Wong, 1963b). It is apparent that of the lower doses of irradiation

the curves are biphasic, the lower melting point being attributable to the helix-to-coil transition of those molecules rendered thermally more labile by the irradiation, and the higher melting point to undamaged collagen molecules or molecules insufficiently damaged to show significant change (Bailey, 1967).

The melting points are given in Table 3, together with the values for $\Delta[\alpha]_T$ and ΔT , the latter being uncorrected for the biphasic nature of the curves. The melting points of the more labile material as a function of irradiation dose are shown in Fig. 8.

DISCUSSION

The action of γ -irradiation may take one or more of the following forms: (a) scission of peptide bonds of the helical structure without destroying the amino acid residues involved in the bonds; (b) scission of peptide bonds of the non-helical telopeptides; (c) destruction of individual amino acid residues; (d) scission of covalent intramolecular bonds; (e) intermolecular cross-linking of collagen molecules.

To discuss the optical-rotation and viscometric data reported in this study, it is necessary to consider the contribution to these physical properties of the various structural elements of the collagen molecule. With optical rotation the primary structure contributes a value equivalent to the residue rotation of collagen in the fully denatured form ($[\alpha]_{365}^{43} - 460^\circ$; Drake, Davison, Bump & Schmitt, 1966), which is the sum of the individual rotations of the amino acids in the molecule plus the structural contribution of the chain. The latter has a value $[\alpha]_{365}^{40} - 27^\circ$ in the total rotation for calf-skin gelatin of $[\alpha]_{365}^{40} - 140^\circ$ (Piez & Gross, 1960) and represents a contribution of approx. 8% in the total rotation for calf-skin of $[\alpha]_{365}^{15} - 340^\circ$. The remaining rotation for collagen, representing some 64% of the total (i.e. from $[\alpha]_{365}^{15} - 450^\circ$ to -1261°), is mainly due to the three helical chains that constitute the molecule, with some contribution from the conformation of these into the triple helix (Hippel & Wong, 1963a). The primary structure, being a random chain, will contribute little to the overall viscosity. Thus Veis & Anesey (1962) give a value of 0.57 dl./g. for the intrinsic viscosity at 40° of bovine γ -gelatin. The secondary structure of collagen will make some contribution to the viscosity, with the tertiary structure making the most contribution owing to the rigidity of the triple helix.

With regard to the current experimental results, the effect of relatively low doses of γ -irradiation is evident in the reduction of specific rotation, and even greater decrease in reduced viscosity of the

irradiated collagen with doses up to about 100 krad, as well as in the relationship between reduced viscosity and collagen concentration (Tables 1 and 2 and Fig. 5). This can be accounted for by a significant decrease in the rigidity of the collagen molecule, which must arise from breaks in one or more of the three individual polypeptide chains of the molecule, without necessarily destroying the amino acid residues involved in the peptide bonds (Cassel, 1959; Bowes & Moss, 1962; Davidson & Cooper, 1968). This will also decrease the helical stability of these chains and the specific rotation of the molecule, but to a smaller extent (Fig. 2). The extent of the recovery of specific rotation (Fig. 1 and Table 1) and of viscosity (Fig. 3 and Table 2) of thermally denatured solutions of the irradiated collagen support these conclusions. The second phase of the irradiation damage occurred above about 100 krad, where a steady decrease in the original and recovered values for specific rotation and reduced viscosity for the unheated and thermally denatured solutions respectively occurred at a lower rate than was the case below this dose range (Tables 1 and 2 and Figs. 2 and 4). The decrease in the rate may be due to the fact that the initial breaks in the polypeptide chains are more effective in decreasing these physical properties than later breaks, a finding that has been reported for other methods of chain scission (Hippel & Wong, 1963b).

These results are different from those of Welling & Bakerman (1964), who concluded from their γ -irradiation of soluble collagen in solution that no change in the optical rotation or viscosity occurred below 90 krad, that above this the viscosity decreased rapidly and that above 130 krad the optical rotation began to fall slightly. The present results (Tables 1 and 2) show that changes in both optical rotation and viscosity were measurable at 1 krad after irradiation of the dry protein, suggesting that the screening effect of the solvent media in protein solutions considerably decreases the effective irradiation dose impinging on the protein. On this basis increased degradation by the 'indirect effect' due to the production of free radicals would appear to be insufficient to offset the protective role of the solvent in decreasing the irradiation intensity.

The γ -irradiation also appears to destroy the ordered polymeric forms of tropocollagen (Davidson & Cooper, 1968), which may account partially for the change in relationship between reduced viscosity and concentration (Bornstein, Kang & Piez, 1966). Extracts of tropocollagen contain covalently linked polymeric molecules, which are reduced initially to monomeric molecules by such agents as sonic treatment and chemical and enzyme treatment (Davison & Drake, 1966; Bornstein *et al.* 1966; Blumenfeld, Rojkind & Gallop, 1965). These linear

polymers may be formed through covalent telopeptide links (Drake *et al.* 1966), and depolymerization could occur through scission of these peptide links.

One of the features of these results is the degree of recovery of optical rotation during mutarotation. Thus the specific-rotation recovery expressed as a percentage of the specific rotation of native collagen ($[\alpha]_{365}^{15} - 1261^\circ$) decreases from 81% to 62% with increased irradiation (Table 1). But the specific-rotation recovery, expressed as a percentage of the specific rotation of the corresponding sample with no thermal history, remains remarkably constant at about 80% for all irradiation doses (Table 1). On the assumption that the sites of irradiation damage can be expected to occur at random in individual molecules, the most general conclusion to be drawn from these observations appears to be that the nucleation sites important in renaturation are comparatively uniformly distributed along the polypeptide chains. Hence fragmentation of each molecule into lengths of damaged and intact structure does not prevent the subsequent nucleation and propagation of the collagen-type structure occurring independently in all the undamaged portions simultaneously, provided that these segments contain the minimum number of residues required to stabilize a length of helical structure (Hippel & Wong, 1963b). A comparatively uniform distribution of nucleating centres would be consistent with the observations of previous workers (Harrington & Hippel, 1961b; Grassmann, Hannig, Endres & Riedel, 1956).

Further, the recovery of a constant fraction of the optical rotation of the original exposed collagen even at high doses suggests that damage is restricted to scission of bonds between α -amino acid residues comprising the 'band' regions of the molecule rather than the 'interband' pyrrolidine-rich regions normally responsible for nucleating the collagen-type structure. The 'interband' regions, which consist largely of the sequence Gly-Pro-X (where X is often Hyp), appear to remain largely intact. It has been estimated that the 'interband' regions represent 60–70% of the collagen molecule and are 20–30 residues long, whereas the 'band' regions are about 15 residues long (Hippel & Wong, 1963b). The stability of these 'interband' regions would explain the relatively high recovery of specific rotation in the gelatin-to-collagen transition, since these regions probably initiate collagen-fold reformation (Hippel & Harrington, 1959; Harrington & Hippel, 1961a; Hippel & Wong, 1963b).

With regard to the data on reduced viscosities (Table 2), it is evident that the γ -irradiation has a greater effect on these than on specific rotation. Thus the reduced viscosity of the irradiated collagen, expressed as a percentage of the reduced viscosity

of native collagen, showed decreases ranging from 68% to 12%. The reduced-viscosity recovery expressed as a percentage of the reduced viscosity of the corresponding unheated sample is fairly constant at about 16%, whereas the reduced-viscosity recovery decreased from 16% to 2.5% with increasing irradiation. Breaks in the polypeptide chains in these 'band' regions would significantly decrease the rigidity of the molecule without affecting the helical content to the same extent.

In the reversion of thermally denatured solutions of the irradiated collagens, it is evident that the relative rates and final recovery values are greater for optical rotation than for viscosity (Tables 1 and 2). In these recovery experiments, the collagen solutions were heated at 45° to convert the tropocollagen, or irradiation-damaged tropocollagen, into individual α - and β -subunits, or fragments of these with the irradiated collagen. Therefore, where fragments of these chains occur in the cooled solutions of sufficient length to re-form individual segments only of the collagen-fold type, optical rotatory power remains high owing to additivity, specific rotation being practically independent of particle size. With reduced viscosity, however, values observed depend closely on hydrodynamic shape and size factors and reduced viscosity will thus be an average value for the various fragments present.

An important feature of the melting curves is their biphasic nature at the lower doses of irradiation, thus giving two denaturation temperatures. Similar results were found by Bailey (1967) from the irradiation of solutions of acid-soluble rat tail collagen between 3 and 30 krad, the lower melting point being due to the thermal disruption of a range of irradiation-damaged triple helices, and the higher melting point to the thermal denaturation of undamaged or marginally damaged triple helices. On this basis, it can be concluded from Fig. 7 that under the experimental conditions employed no intact collagen-type structure remains at doses above 100 krad, corresponding to the disappearance of the high-temperature 'shoulder' region on the melting curve. For solutions of acid-soluble collagen Bailey (1967) found that at 30 krad all the molecules were sufficiently damaged to be thermally labile below the melting point of the native collagen. In view of the different sources of collagen, quantitative differences in the amounts of irradiation required to produce comparable effects in the present study and in previous studies by other workers would appear to be due to the protective screening action of the solvent environment in the latter cases.

The melting curves (Fig. 7) give melting points for the damaged collagen that decrease rapidly

with the initial low doses of irradiation, and then decrease at a much less rapid rate as the irradiation is increased (Fig. 8), which is consistent with a progressive loss in helical structure. The quantity $\Delta[\alpha]_T$ is proportional to the number of residues that pass from a helical to a random-coil conformation and, as this value decreases as the irradiation dose is increased (Table 3), this would indicate a decrease in the helical content. Similarly the temperature difference (ΔT), which is directly related to the phase transition and serves as a convenient measure of the 'degree of co-operativeness' of the helical structure (Hippel & Wong, 1963b), increases in proportion to the irradiation (Table 3).

Bailey, Rhodes & Cater (1964), Bailey (1967) and Tomoda & Tsuda (1961) have shown that γ -rays can introduce intermolecular cross-links into collagen fibrils, soluble collagen and gelatin in the presence of water, but this tendency is decreased in the presence of oxygen and radical scavengers (Welling & Bakerman, 1964), suggesting that these cross-links appear to arise from the action of free radicals. In some of the experimental results reported here, it is evident that those for the dose range 5 krad-2.6 Mrads do not always fall into line with the rest of the results (see e.g. Figs. 4 and 5). It is possible therefore that in this range some cross-links are formed on irradiating dry collagen by a mechanism other than that involving the action of free radicals derived from the solvent.

In conclusion, the irradiation damage is not reversible, unlike the heat-denaturation of collagen, which is at least partially reversible (see e.g. Beier & Engel, 1966; Drake & Veis, 1964), since the optical rotation and viscosity of solutions of irradiated collagen kept at 15° without any heating step remained constant over periods up to 30 hr. and showed no tendency to recover the values of the native soluble collagen. This is further shown by the decrease in mutarotation and viscosity recovery at 15° for thermally denatured solutions of collagen treated with increasing doses of irradiation.

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REFERENCES

- Bailey, A. J. (1967). *Radiation Res.* **31**, 306.
 Bailey, A. J., Rhodes, D. N. & Cater, C. W. (1964). *Radiation Res.* **22**, 606.
 Beier, G. & Engel, J. (1966). *Biochemistry*, **5**, 2744.
 Blumenfeld, O. O., Rojkind, M. & Gallop, P. M. (1965). *Biochemistry*, **4**, 1780.
 Bornstein, P., Kang, A. H. & Piez, K. A. (1966). *Biochemistry*, **5**, 3803.
 Bowes, J. H. & Moss, J. A. (1962). *Radiation Res.* **16**, 211.
 Cassel, J. (1959). *J. Amer. Leath. Chem. Ass.* **54**, 8.
 Cooper, D. R. & Davidson, R. J. (1965). *Biochem. J.* **97**, 139.
 Copeland, E. S., Sanner, T. & Pihl, A. (1968). *Radiation Res.* **35**, 437.
 Davidson, R. J. & Cooper, D. R. (1967). *Biochem. J.* **105**, 965.
 Davidson, R. J. & Cooper, D. R. (1968). *Biochem. J.* **107**, 29.
 Davison, P. F. & Drake, M. P. (1966). *Biochemistry*, **5**, 313.
 Drake, M. P., Davison, P. F., Bump, S. & Schmitt, F. O. (1966). *Biochemistry*, **5**, 301.
 Drake, M. P. & Veis, A. (1964). *Biochemistry*, **3**, 135.
 Grassmann, W., Hannig, K., Endres, H. & Riedel, A. (1956). *Hoppe-Seyl. Z.* **306**, 123.
 Harrington, W. F. & Hippel, P. H. von (1961a). *Advanc. Protein Chem.* **16**, 1.
 Harrington, W. F. & Hippel, P. H. von (1961b). *Arch. Biochem. Biophys.* **92**, 100.
 Hippel, P. H. von & Harrington, W. F. (1959). *Biochim. biophys. Acta*, **36**, 427.
 Hippel, P. H. von & Wong, K. Y. (1963a). *Biochemistry*, **2**, 1387.
 Hippel, P. H. von & Wong, K. Y. (1963b). *Biochemistry*, **2**, 1399.
 Piez, K. A. & Gross, J. (1960). *J. biol. Chem.* **235**, 995.
 Tomoda, Y. & Tsuda, M. (1961). *Nature, Lond.*, **190**, 905.
 Veis, A. & Anesey, J. (1962). *Arch. Biochem. Biophys.* **98**, 104.
 Welling, L. & Bakerman, S. (1964). *Nature, Lond.*, **201**, 495.
 Yang, J. T. (1961). *Advanc. Protein Chem.* **16**, 323.