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X-ray diffraction studies of rennin crystals

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Rennin, purified from commercial rennet powder by Foltmann's method (Foltmann 1959) and precipitated from solution by sodium chloride, crystallizes in the form of rectangular blocks, often nearly equidimensional but sometimes elongated, usually along the direction of highest refractive index. X-ray diffraction photographs (figure 1 is an example) show that the symmetry is orthorhombic and the lattice body-centred. Essential information is given in table 1.

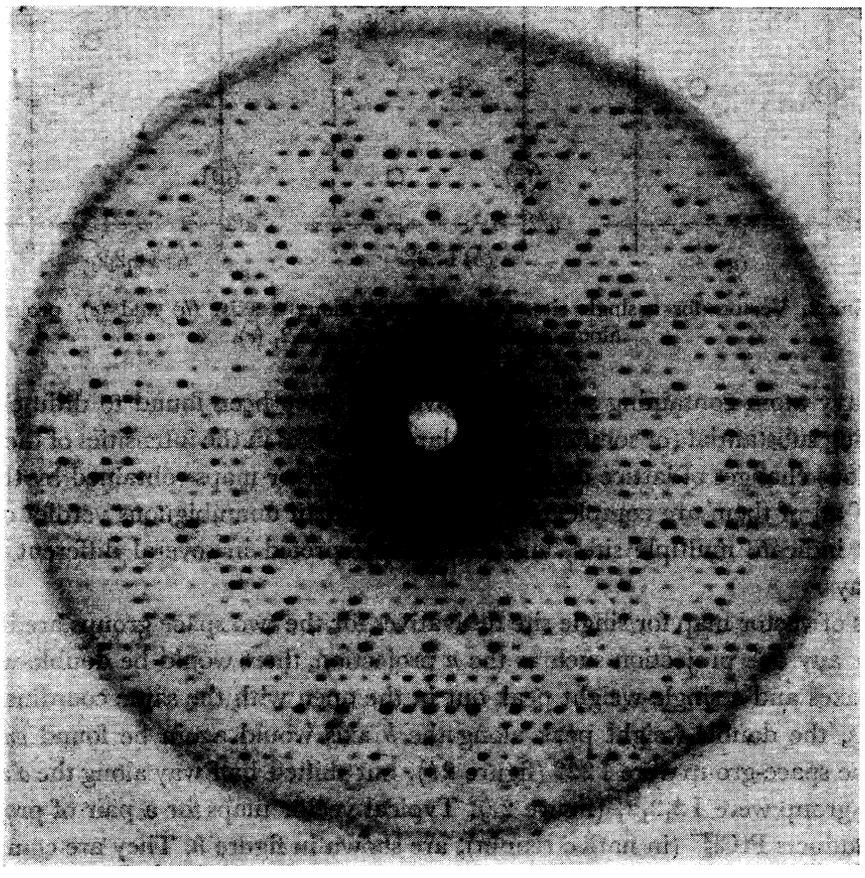

Figure 1. ($hk0$) precession photograph of crosslinked rennin. The outer edge of this photograph corresponds to a spacing of approximately 0.3 nm.

The two space-groups I 222 and I $2_12_12_1$ have the same symmetry elements—twofold rotation axes and twofold screw axes—but arranged in different ways; in I 222 the twofold rotation axes in the three principal directions intersect, while in I $2_12_12_1$ they do not. It is not possible to distinguish between these arrangements by the usual methods, based on types of absent reflexions or intensity statistics; but if a heavy atom derivative or adduct having one heavy atom

TABLE 1

		a_0/nm	b_0/nm	c_0/nm
unit cell dimensions:	native	7.97	11.38	7.28
	crosslinked	7.99	11.41	7.27

Density 1.243 g cm^{-3}. Water content $\sim$ 50 %
Space-group I 222 or I $2_12_12_1$. Eight molecules per unit cell, four per lattice point, one per asymmetric unit. All three principal projections centrosymmetric.

per protein molecule were available, the correct arrangement would be clearly indicated by vector maps of two (or better still, three) principal projections (Rogers 1950). Simple heavy atom derivatives or adducts are, of course, necessary to open the way to a solution of the protein structure, and therefore the space group ambiguity is not really an additional difficulty. Everything depends on finding simple heavy atom derivatives or adducts.

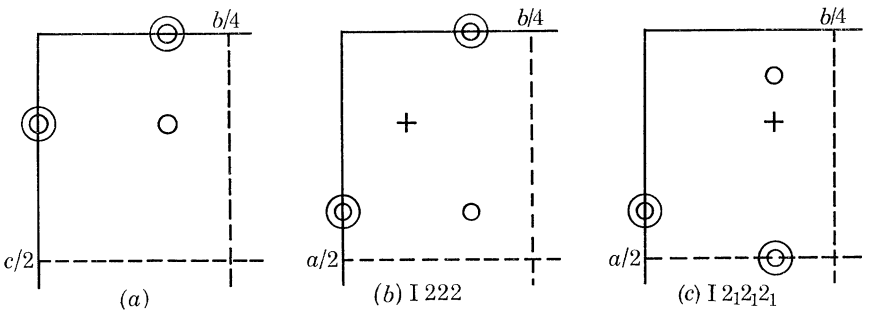

FIGURE 2. Vectors for a single site. (*a*), projection along *a* axis; (*b*) and (*c*), projection along *c* for I 222 (*b*) and I $2_12_12_1$ (*c*).

Many heavy atom containing substances (over 30) have been found to diffuse into rennin crystals, giving substantial (or sometimes very large) changes in the intensities of the diffractions without serious changes of lattice dimensions; but the vector maps (obtained by the Patterson synthesis) of all of them are complex; they do not give an unambiguous verdict on the space group, they indicate multiple sites, and can be interpreted in several different but equally plausible ways.

The types of vector map for single site derivatives for the two space groups are illustrated in figure 2; for any one projection such as the *a* projection, there would be double-weight peaks along both axes and a single-weight peak out in the open with the same coordinates. For the *c* projections, the double-weight peak along the *b* axis would again be found *on* the *b* axis ($x = 0$) if the space-group were I 222 (figure 2*b*), but shifted half way along the *a* axis ($x = \frac{1}{2}$) if the space group were I $2_12_12_1$ (figure 2*c*). Typical vector maps for a pair of projections, for one of the adducts $PtCl_6^{2-}$ (in native rennin), are shown in figure 3. They are complex and do not settle the space group unambiguously. Moreover, several interpretations are possible. Since all the heavy atom substances tried so far give vector maps of at least equal complexity, many attempts have been made to influence the heavy atom uptake by varying salt concentration or pH, or adding other substances.

Much of the work has been done with rennin crosslinked by treatment with 1 % glutaraldehyde (Quiocho & Richards 1964; Richards & Knowles 1968). Crosslinking confers several advantages: the crystals, which are normally very fragile, become much more robust, so that

there is much less chance of damage in handling; they become completely insoluble but remain permeable to solutions, so that NaCl can be washed out giving salt-free crystals, or replaced by other substances; and the pH can be varied over a wide range without dissolution or deterioration. These properties very much extend the scope of experiments aimed at influencing the uptake of heavy atom containing substances; owing to the complexities of heavy atom effects already referred to, many experiments have been done in the hope of reducing the number of sites. Crosslinking causes some changes of the intensities of diffraction but only slight changes of lattice dimensions and no deterioration of quality; the range of diffraction extends out to

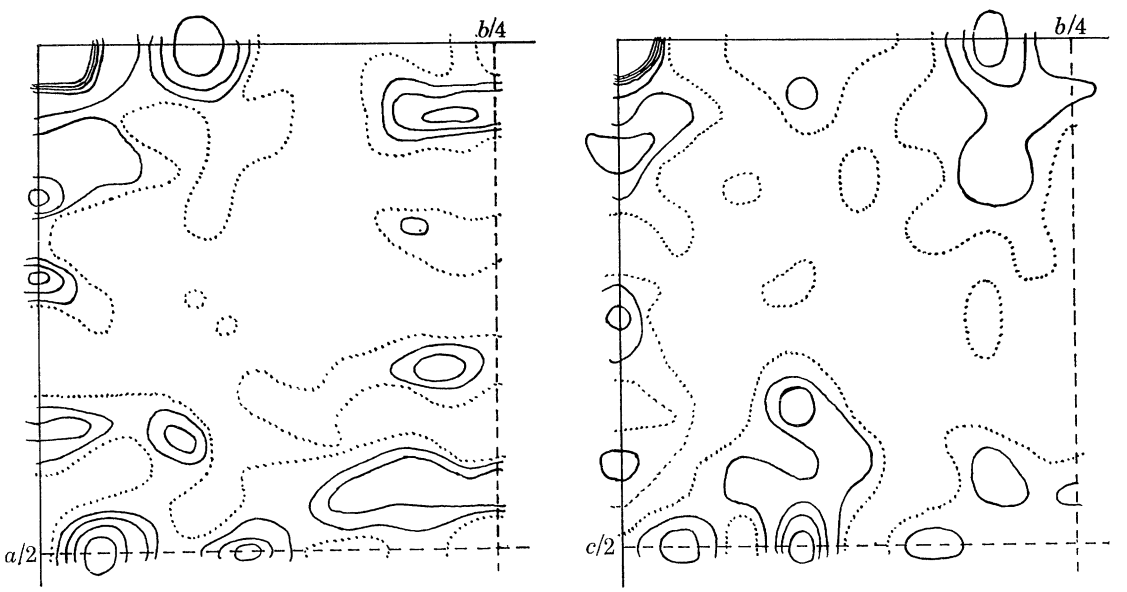

FIGURE 3. Difference Patterson projections for native rennin substituted with 5 mmol l^{-1} of $PtCl_6^{2-}$ (with 2.5 mol l^{-1} of NaCl at pH 6). The resolution is 0.55 nm.

0.2 nm. It has been found that all the factors mentioned—salt concentration, pH, and the addition of other substances such as amino acids—influence greatly the amount of heavy atom taken up by the crystal (as indicated by the changes of diffraction intensities). Examples are shown in tables 2 and 3. Increasing salt concentration sometimes reduces the uptake without appreciably changing the vector map, suggesting that the occupancies of all the heavy atom sites are reduced in about the same ratio; but in other cases there is a considerable change in the vector map without much change in the uptake, suggesting that the occupancies of some sites are reduced while those of others are increased. Changes in pH have very large effects;

TABLE 2. INFLUENCE OF SALTS AND AMINO ACIDS ON HEAVY ATOM UPTAKE $\left(\frac{\Sigma|F_{PH}-F_P|}{\Sigma|F_P|}\right)$ IN CROSSLINKED RENNIN CRYSTALS AT pH 6

	salt free	salt in solution	amino acid in solution
Yb^{3+}	40	(0.5 mol l^{-1} of NaCl), *ca.* 20 (2 mol l^{-1} of NaCl), *ca.* 0	(2:1 L-Trp), 35
$PtCl_6^{2-}$	40	(2 mol l^{-1} of NaCl), 40 (2.5 mmol l^{-1} of $NaNO_2$), *ca.* 0	(2:1 L-His), *ca.* 21
diacetoxy-mercurithymol	disorder	(0.5 mol l^{-1} of NaCl), *ca.* 0	(10:1 Gly), *ca.* 22

for negative ions, the uptake increases with decreasing pH, and at pH 2 there may be (as in the case of $PtCl_6^{2-}$, table 3) enormous changes in diffraction intensities accompanied by disorder in the crystal, shown by much weakened high order diffractions; for positive ions, the reverse is true. Mercury compounds do not always fit into this scheme: there may be special effects. None of these experiments have produced the desired drastic simplification of the vector maps to the point where unambiguous interpretation in terms of one or very few sites is possible; but an apparent partial simplification has been produced in some cases.

TABLE 3. INFLUENCE OF pH ON HEAVY ATOM UPTAKE $\left(\frac{\Sigma|F_{PH}-F_P|}{\Sigma|F_P|}\right)$ IN SALT FREE CROSSLINKED RENNIN CRYSTALS

	pH 2	pH 6	pH 8.7
$PtCl_6^{2-}$	disorder	40	21
I^-	20	*ca.* 0	—
WO_4^{2-}	21	*ca.* 0	—
Yb^{3+}	*ca.* 0	40	—
Ag^+	*ca.* 0	40	disorder
diacetoxy-mercuri-thymol	*ca.* 0	disorder	—

Many possible interpretations of the simpler vector maps have been considered. The criteria of correctness are, first, that sites suggested by the vector maps should account reasonably well for all the large structure amplitudes; secondly that the three principal projections should indicate consistent sites; and thirdly that the results from different heavy atom patterns should lead to the same information about the phases of protein structure amplitudes. Owing to the doubt about the space group, the first approach was to try to solve one principal projection for several heavy atom adducts (at 0.6 nm resolution) aiming at consistency of protein phases, then to solve independently a second projection for the same set of heavy atom adducts, again attaining as much consistency of protein phases as possible, and subsequently to compare the projections to see whether the heavy atom sites were consistent for either of the space groups. Six heavy atom adducts were used, and for each projection an encouraging degree of consistency of protein phases was obtained; but the sites in the two projections were not sufficiently consistent for either of the space groups. Evidently the solutions proposed for one or both of the projections were incorrect.

The failure of this approach may have been due to insufficient data points at 0.6 nm resolution (about 100); for multi-site adducts, a larger number of data points is probably necessary. One way of providing a much larger number of data is to extend diffraction measurements to three dimensions; this was done for one of the adducts ($YbCl_3$), giving 1300 data points. A three-dimensional vector map calculated by the Patterson synthesis $\Sigma(F_{PH}-F_P)^2$ is inevitably not a geniune heavy atom vector map. Owing to the difference of phase between F_{PH} and F_P for the general diffractions $(F_{PH}-F_P)$ does not equal F_H; nevertheless, heavy atom vectors are expected to be prominent. Moreover, self vectors (those between symmetry related atoms) are to be found on the faces of the unit cell and at half-way planes, while cross-vectors (those between atoms not related by symmetry) are distributed in space. The map resulting from this synthesis presents problems. The principal discrete peaks do occur on cell faces and halfway planes, and suggest two, or perhaps three, sites in I222, but structure amplitudes calculated

for these sites do not agree well with measured values and little refinement occurred on adjusting coordinates and occupancies. This was not entirely surprising, for some strong features of the vector map are not accounted for—in particular, a strong, almost continuous column along the *c* axis. If this is not due to the imperfection arising from the phase differences, it suggests sites rather closely spaced along *c*. Alternatively, the fact that this feature is also present in the three-dimensional vector map of crosslinked rennin itself suggests that it may mean lack of molecular isomorphism.

Another way of providing more data is to go to higher resolution. Vector maps at 0.35 nm resolution for $YbCl_3$ and for a quite different adduct, K_2PtCl_6, have been obtained, for the three principal projections. Each map by itself permits more than one interpretation; they are now being considered with the aim of finding sets of heavy atom sites which are consistent for all three projections and lead to the same protein phases. At the same time, we continue to try other heavy atom containing substances in the search for a really simple adduct, and to investigate the modification of heavy atom uptake by other dissolved substances and change of pH. In the course of this work we have amassed a great deal of evidence on the competition of molecules for sites on the protein molecule; the meaning of this will not be clear until we know the protein structure.

Our experiences with rennin raise an interesting problem, when compared with those encountered with the several other proteins whose structures have been solved because simple heavy atom adducts were discovered. The comparison with pepsin is especially striking; rennin and pepsin are closely related enzymes, both in biochemical function and in molecular structure, as far as present knowledge goes, yet simple heavy atom adducts of pepsin have been found (Bakulina, Borisov, Melik-Adamyan, Shutskever & Andreeva 1968), while the same heavy atom ions (such as $PtCl_4^{2-}$ and HgI_3^-) give complex adducts with rennin. Comparison of the numbers of the different amino acid units shows one striking difference; the number of positively charged side groups which would be expected to attract the negatively charged heavy atom ions, is much smaller in pepsin than in rennin (pepsin: 1 Lys, 1 His, 2 Arg; rennin: 7 or 8 Lys, 4 His and 5 Arg). This seems a possible explanation of the greater complexity of the rennin adducts, yet in several other proteins giving simple adducts there are at least as many positively charged groups as in rennin; possibly some of these positive groups are in less accessible positions.

Another comparison which invites consideration is based on the sizes of the protein molecules. It would be expected that the larger the protein molecule, the greater the number of suitable sites for heavy atom ions; yet several proteins with molecular weights between 17 000 and 35 000 have been found to form simple heavy atom adducts, while for the smaller molecules insulin and ribonuclease it has been difficult to find simple adducts. No doubt these facts will not be understood in any detail until more protein structures are known, but any generalizations emerging from those already known would be interesting and perhaps helpful to those wishing to study new protein structures.

Some of the complex effects found in rennin and some other proteins may perhaps be due, not to multiple heavy atom sites but to changes of protein conformation. Constancy of lattice dimensions is no guarantee of molecular isomorphism; parts of the chain may swing into different positions when certain ions attach themselves, and such changes may be responsible for part of the change of the X-ray diffraction pattern. It is not easy to obtain clear evidence on this point.

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References (Bunn *et al.*)

Bakulina, V. M., Borisov, V. V., Melik-Adamyan, V. R., Shutskever, N. E. & Andreeva, N. S. 1968 *Kristallografiya* **13**, 33.
Foltmann, B. 1959 *Acta Chem. Scand.* **13**, 1927.
Quiocho, F. A. & Richards, F. M. 1964 *Proc. natn. Acad. Sci. U.S.A.* **52**, 833.
Richards, F. M. & Knowles, J. R. 1968 *J. molec. Biol.* **37**, 231.
Rogers, D. 1950 *Acta Crystallogr.* **3**, 455.

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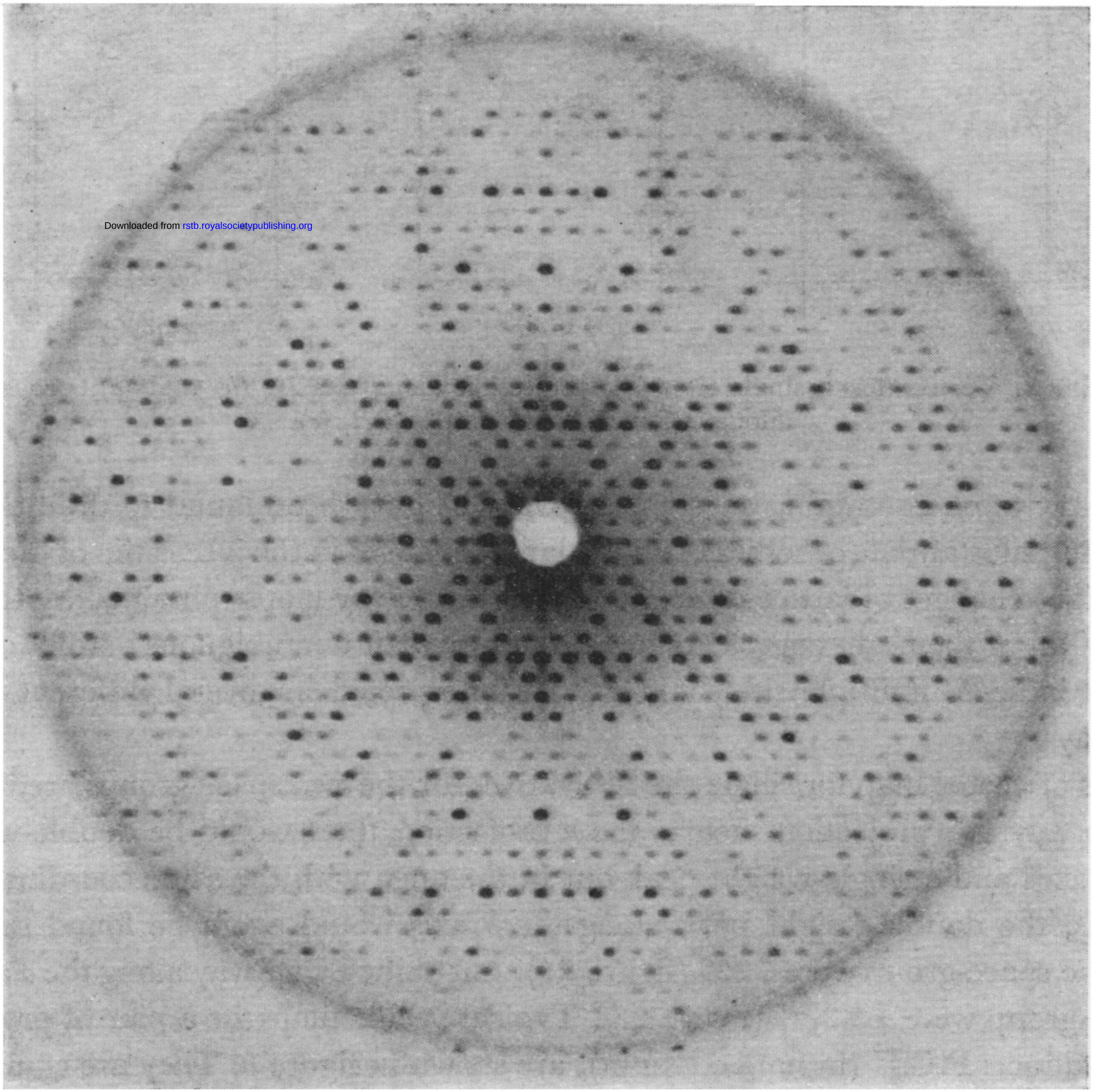

FIGURE 1. $(hk0)$ precession photograph of crosslinked rennin. The outer edge of this photograph corresponds to a spacing of approximately 0.3 nm.

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