determine peak positions to 0·01°, therefore a change in separation of 0·02° may be detected, or a change in $\lambda$ (or a) of about 0·0003 Å. This would be the order of the expected error in any series of measurements, just from resolution considerations. The crystal may be aligned as precisely as desired, since many peaks are available. Fig. 10 summarizes the values of $(a/\lambda)$ obtained for this Ge crystal and Cu $K\alpha_1$ radiation, using the Renninger peaks shown in the figure (on the plot of $(a/\lambda)$ versus $\alpha$). We feel that the measured value of $(a/\lambda)$ is significantly different from the accepted value, even if a refraction correction is applied; however, no attempt has been made to analyze the experiment any further than indicated here.

The authors would like to acknowledge the programming done by W. W. O'Neill to permit CRT output of the IBM 704 for tracing the curves.

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# Crystalline forms of Bovine Pancreatic Ribonuclease. Some new Modifications

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(*Received* 15 *December* 1959 *and in revised form* 21 *February* 1961 *and* 5 *June* 1961)

Seven new crystalline forms of bovine pancreatic ribonuclease have been prepared and characterized by X-ray diffraction. One of these forms (XI) is obtained in the vicinity of the isoelectric point of the protein. Some are metal derivatives: VIII (Cu), X (Ni), XII (Hg). Two of the forms are obtained from aqueous methyl alcohol at different temperatures: IX and XIV. One of the forms (XII) is obtained from ribonuclease which had been treated with tribromoacetic anhydride.

## Preparative techniques and materials

The techniques used in preparation of the seven new crystalline modifications of bovine pancreatic ribonuclease described here are all based on the methods outlined in a preceding paper (King, Magdoff, Adelman, and Harker, 1956; hereinafter denoted by KMAH). The letter designations applied in KMAH to the various methods of crystallization will be continued here also. The variations involve addition of metallic compounds, choise of solvent, pH control, protein-modification reactions, and temperature, and are described below in the sections devoted to the respective crystalline modifications.

The ribonuclease used in these studies was obtained from Armour Laboratories, lot numbers 381–059 and 647–213. (For convenience, ribonuclease may be abbreviated: RNase.) The solvents and reagents used were the purest commercially available, except that the solvent 2-methyl-2,4-pentanediol (abbreviated hereinafter as MPD) was further purified by adsorption of impurities on a cation-exchange column and distillation in vacuo from $K_2HPO_4$. When thus purified, MPD shows advantages over the simple alcohols as a medium for crystallizing RNase because of its lower tendency to induce alterations in the protein and its lower volatility.

Compositions of solutions are expressed below as volume percent of organic solvent in the final solution; it is understood that $x$ volume percent of a solvent implies a mixture of $x$ volumes solvent with $(100-x)$ volumes water. In samples prepared by method ($D$), addition of metal compounds and adjustment of pH are performed prior to freezing and addition of the

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Table 1. *Details of preparation and crystallographic data for the new modifications of ribonuclease*

| Unit cell no. | Details of preparation | $a$ (Å) | $b$ (Å) | $c$ (Å) | $\beta$ (°) | Unit cell volume (Å$^3$) |
|---|---|---|---|---|---|---|
| | Modification VIII, space group $C2$, $Z=4$ | | | | | |
| 31 | 55 vol.% MPD, pH 5·51, 1 $Cu^{++}$ per RNase, Method ($D$) | 58·65 | 53·92 | 43·40 | 119·65 | 119 000 |
| | Modification IX, space group $P2_12_12_1$, $Z=4$ | | | | | |
| 32 | 60 vol.% $Me$OH, Method ($B$); soaked in 75 vol.% $Me$OH | 42 | 53 | 46 | — | 101,000 |
| 33 | 60 vol.% $Me$OH, Method ($B$); air-dried | 40 | 53 | 35 | — | 76,000 |
| | Modification $X$, space group $P2_1$, $Z=4$ | | | | | |
| 34 | 55 vol.% MPD, pH 6·08, 1 $Ni^{++}$ per RNase, Method ($D$) | 50·00 | 74·58 | 31·21 | 94 | 116,100 |
| | Modification XI, space group $P2_12_12_1$, $Z=4$ | | | | | |
| 35 | Deionized RNase (isoionic, pH ~ 9·5) 55 vol.% MPD, Method ($D$); soaked in 75 vol.% MPD | 42·47 | 74·28 | 38·26 | — | 120,700 |
| | Modification XII, space group $P2_12_12_1$(?), $Z=8$ | | | | | |
| 36 | Treated with 1 $Hg(OAc)_2$ per RNase at 3–5 °C. one month, dialyzed, lyophilized, crystallized by Method ($B$) from 55 vol.% MPD | 89·0 | 92·4 | 34·4 | — | 283,000 |
| | Modification XIII, space group $P2_1$, $Z=4$ | | | | | |
| 37 | Treated with 7½ $(CBr_3CO)_2O$ per RNase, adjusted to pH7, dialyzed, lyophilized, crystallized by Method ($B$) from 55 vol.% MPD | 51·35 | 76·00 | 31·38 | 106·11 | 117,700 |
| | Modification XIV, space group $P2_12_12$(?), $Z=4$ | | | | | |
| 38 | 55 vol.% $Me$OH, 3–5 °C., Method ($B$) | 44 | 75 | 39 | — | 129,000 |

solvent. Thus, the cited pH values are those of the aqueous solutions, not the final mixtures.

The growth of the crystals usually took two to six months, except that modification XI generally required only one or two weeks. Crystals of longest dimension 0·25–0·5 mm. were generally preferred for X-ray study, although some modifications could only be grown in smaller sizes. Since crystals in some preparations were often unstable in their mother liquors, but stable in media containing less water by virtue of their lower solubility therein, the practice was generally made of transferring crystals after growth had ceased into 'preserving solutions' containing about 25 volume percent water. In such cases, the compositions of both the crystallization and the preserving media are given.

### X-ray diffraction techniques

X-ray data were taken on the Geiger-counter diffractometer as described by Furnas and Harker (1955); precession photographs were also taken of form XIII, verifying the monoclinic symmetry. Otherwise, the space groups were determined as outlined here. First, the lattice of each crystal was identified by the method described by Furnas (1957). In this process, the central sphere of data in reciprocal space, comprising spacings greater than 6 Å, was scanned. Edges and diagonals of the reciprocal-lattice nets were searched for intermediate reflections, and the existence of possible symmetry elements was tested by systematic intensity measurements. Then a number of selected central rows was scanned in $2\theta$ out to the highest angles at which reflections could be observed. These rows were selected such as to test thoroughly for screw-axis extinctions (glide planes being impossible in protein crystals), to verify the existence of the rotation axes, to search for reflections violating the assigned lattice, as well as to ascertain the minimum observable spacing.

The number $Z$ of protein molecules per unit cell was generally estimated without recourse to density data from considerations of the volume per asymmetric unit in the various modifications. The volume of the anhydrous RNase molecule, as calculated from the partial specific volume (Harrington and Schellman, 1956) and the molecular weight (Hirs, Moore, and Stein, 1956) is 15,800 Å$^3$. Now, most of the modifications show volumes per asymmetric unit closely ranging about 30,000 Å$^3$. Hence, we must assign one molecule only to the asymmetric unit in these forms, or make the implausible assumption that the crystals are nearly anhydrous. The remaining forms, X, XII, and XIII, as well as forms III and IV studied in KMAH, show volumes per asymmetric unit (V./as. u.) ranging from 58,000 to 70,800 Å$^3$. Thus, by assigning two RNase molecules to the asymmetric unit in these forms, we arrive at a consistent set of solvated molecular volumes for all modifications, ranging from 25,200 to 35,400 Å$^3$.

The lattice parameters and detailed conditions of preparation of the crystals studied are given in Table 1. A comparison is given in Table 2 of the volumes per asymmetric unit (V./as.u.), per molecule (V./molec.), and per molecular-weight unit (V./m.w.u.) of the present and previously-studied forms, together with the calculated volume fractions of solvent in the crystals. Each modification is represented here by

Table 2. *A comparison of the packing of ribonuclease molecules in the various crystalline modifications.*

| Modification | Unit cell no. | V./as.u. | V./molec. | V./m.w.u. | Vol.% solvent |
|---|---|---|---|---|---|
| I | 2 | 31,800 | 31,800 | 2·32 | 50 |
| II | 22 | 29,800 | 29,800 | 2·18 | 47 |
| III | 24 | 68,400 | 34,200 | 2·50 | 54 |
| IV | 25 | 63,400 | 31,700 | 2·32 | 50 |
| V | 27 | 29,900 | 29,900 | 2·18 | 47 |
| VI | 29 | 34,500 | 34,500 | 2·52 | 54 |
| VII | 30 | 29,000 | 29,000 | 2·12 | 46 |
| VIII | 31 | 29,800 | 29,800 | 2·18 | 47 |
| IX | 32 | 25,200 | 25,200 | 1·84 | 37 |
| X | 34 | 58,000 | 29,000 | 2·12 | 46 |
| XI | 35 | 30,200 | 30,200 | 2·21 | 48 |
| XII | 36 | 70,800 | 35,400 | 2·59 | 55 |
| XIII | 37 | 58,600 | 29,400 | 2·15 | 46 |
| XIV | 38 | 32,200 | 32,200 | 2·35 | 51 |

some typical unit cell, denoted by its unit-cell number as given in Table 1 or in Table 1 of KMAH.

## Description of the crystal modifications

*Ribonuclease VIII*

This modification is a complex of RNase with copper. It is the form usually observed in copper-containing samples, although form I is also observed in these samples occasionally. Crystals of form VIII are bright blue in color, as the crystals of form I obtained in the presence of copper are, or indeed, as crystals of any of the modifications become upon soaking in copper-containing media. Form VIII is monoclinic with space group $C2$, with four molecules in the unit cell. The initial Cu:RNase ratios in the samples in which this form appeared were from one to three. The pH range was about 4·8–5·5, although these are not necessarily the limiting pH values. The crystals are highly fragile plates flattened on (010) and elongated along $c$. They usually occur in complicated intergrowths, but are occasionally single. Irregularities in the other faces have thus far prevented a complete study of the morphology. The minumum spacing observed in diffraction is 2·7 Å.

This modification has thus far been prepared only from aqueous MPD solutions, since similar experiments in aqueous alcohol gave no crystals. The copper in the crystals is evidently firmly bound, since no loss or change of color occurs upon soaking the crystals in dimethylglyoxime solution (in 75 volume % MPD), as occurs with other modifications of RNase into which copper has been introduced by soaking in copper solutions (such copper solutions are made up in 75 volume % MPD to prevent re-solution of the RNase crystals). Reagents with more affinity for copper, however, do extract this metal from crystals of form VIII, *e.g.*, the crystals are decolorized by ethylenediaminetetraacetic acid.

*Ribonuclease IX*

This form is orthorhombic, space group $P2_12_12_1$, with four molecules in the unit cell. This cell has a volume 20% smaller than that of form I. This packing, in which only 37% of the volume is occupied by solvent, is the most compact thus far found for wet RNase crystals. This form is obtained only from 55–60 volume % methyl alcohol, and then only by use of fresh alcohol solutions which have not been exposed unduly to light and air. This observation suggests that products of photooxidation of methyl alcohol, e.g., formaldehyde, may interfere with the crystallization. The crystals are generally fine laths elongated along $a$, only rarely large enough for X-ray studies. Thus, the best available diffractometer patterns are very faint. Nevertheless, it is of interest that the patterns do not deteriorate when the crystals are dried, the minimum observed spacing for both the wet and the dry crystal being about 2·8 Å.

*Ribonuclease X*

This monoclinic modification is another nickel complex, which has been found growing sporadically in samples together with form I. The space group is $P2_1$, with four melocules in the unit cell. The crystals are laths appearing in intergrowths, but single-crystal fragments may be cleaved off for X-ray study. The cleavage plane is (001), so that the cleavage fragment studied was bounded by the forms {100}, {$hk0$}, and {001}. This form is metastable, reverting to form I if left in the mother liquor.

*Ribonuclease XI*

This modification is observed at pH values near the isoelectric point of the protein (about pH 9·5) under a wide variety of conditions. This form is apparently very stable, and shows a high degree of crystalline perfection, the minimum observed spacing being 1·8 Å. With its stability, crystalline perfection, and insensitivity to exact maintenance of physicochemical conditions, this form plays a role in the alkaline range similar to that played by RNase II in the neutral to acid range. The transition $p_H$ between these two forms appears to be 8·7.

Modification XI is orthorhombic, space group $P2_12_12_1$, whith four molecules in a cell of dimensions not greatly different form that of form I. However, a strikingly different distribution of intensities serves to distinguish it from form I. The crystals are stout needles of rather irregular outline, although they generally give X-ray patterns of good single crystals. The direction of elongation is $a$. Perfect cleavage on (100) is observed, so that fragments of suitable size for X-ray study may be easily obtained.

Crystals of form XI are obtained in the pH range above 8·7, regardless of whether the pH is adjusted by adding NaOH or an organic amine (N,N-dimethylbenzylamine), or by deionizing the RNase solution with a mixed-bed ion-exchanger, although the size of crystals obtained is somewhat sensitive to the method of adjusting the pH.

*Ribonuclease XII*

This orthorhombic modification is a derivative of RNase prepared by reaction with mercuric acetate in equimolar ratio. It shows a diffraction pattern consistent with the space group $P2_12_12_1$. However, the diffraction data are limited, the only $00l$ orders shown being 002 and 004. Thus, the establishment of the $c$ screw-axis is not certain. From the observed cell volume, there should be two molecules in the asymmetric unit, or eight in the unit cell. The crystals are small needles elongated along $c$, giving only weak X-ray patterns. While form XII is a disappointing object for X-ray studies, it is of chemical interest in that it is grown from material containing very firmly bound, non-dialyzable mercury, in spite of the absence of SH groups in RNase.

*Ribonuclease XIII*

This monoclinic modification was obtained from RNase which had been treated with $7\frac{1}{2}$ moles tribromoacetic anhydride per mole of RNase at pH 8·5. The product has not been characterized chemically. The space group is $P2_1$, with four molecules in the unit cell. The crystals are lozenges with the $b$ axis lying along the bisector of the acute angles. The crystals obtained are of good quality and size for X-ray studies, but the reproducibility of the preparation and range of stability are in doubt, since other RNase samples treated with either smaller or larger proportions of this anhydride gave only modification II.

*Ribonuclease XIV*

This form has been obtained only from aqueous methyl alcohol at refrigerator temperatures in the form of rectangular plates. While the preparation is reproducible, form XIV is metastable even at these temperatures, reverting to modification II after some months. The crystals were not stabilized by soaking them in 75 volume % methyl alcohol, but broke up in this medium. At room temperature, form XIV is quite unstable, so that only a partial survey of the reciprocal lattice could be made without the use of low-temperature apparatus. This survey suggested the space group $P2_12_12$, with four molecules in the unit cell, but thorough verification was impossible.

It is of interest that two of the new modifications, IX and XIV, have been produced by precipitation with methyl alcohol, while most organic solvents at pH $\sim 5$ without other additions give only forms II, III, or IV. This may be considered as evidence that methyl alcohol interacts with RNase in a way differing from that exhibited by other solvents. This specific interaction is analogous with that responsible for the crystallization of form V from solutions containing 1,3-propanediol or $\gamma$-butyrolactone. However, since form II can also be obtained from aqueous methyl alcohol, the specific difference cannot be great.

The authors wish to thank Mrs Virginia Hong Wei, Miss Menilyn Merrick, Mr Edward Thomas Bisiakowski, and Mr Edward J. Mordia for valuable assistance in the chemical work involved in these studies.

It is also a pleasure for the authors to express their appreciation to the several institutions the continued support of which has made possible the work of the Protein Structure Project. They are: The Dean Langmuir Foundation, The Rockefeller Foundation, The Damon Runyon Memorial Foundation, The New York Foundation, The Polytechnic Institute of Brooklyn, and the International Business Machines Corporation.

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