The Inactivation of Ribonuclease during the Isolation of Ribonucleic Acids and Ribonucleoproteins from Yeast

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The isolation of ribonucleic acids and ribonucleoproteins in an undegraded state is complicated by the presence of degradative enzymes, particularly ribonuclease. It has been particularly difficult to find an inhibitor for this enzyme that was suitable for use in preparative work on the isolation of ribonucleoproteins. Inhibitors such as sodium cholate (Jones, Marsh & Rizvi, 1957) and sodium dodecyl sulphate (Crestfield, Smith & Allen, 1955), which have been used for the isolation of ribonucleic acids, cannot be used for the isolation of ribonucleoproteins because of their possible denaturing action on the proteins. Polyacidic substances have been used to inhibit pancreatic ribonuclease (Zöllner & Fellig, 1953; Vandendriessche, 1956) and, although these could possibly be used in the isolation of ribonucleoproteins, the removal of the inhibitor from the final product would be difficult. Copper and Zn^{2+} ions inhibit pancreatic ribonuclease (Zittle, 1946; Davis & Allen, 1955) but, as the present investigations have shown, these have limited use in isolation procedures for ribonucleic acids and ribonucleoproteins. The object of the present work was to find inhibitors suitable for this purpose.

EXPERIMENTAL

Nitrogen was determined by Belcher & Bhatty's (1956) method and phosphorus as described by Jones, Lee & Peacocke (1951). Protein (5–100 μg.) was estimated by the use of the Folin–Ciocalteu reagent as described by Lowry, Rosebrough, Farr & Randall (1951). Crystalline ovalbumin was used as the standard. Analyses were performed on moist material and corrected for moisture contents determined on separate samples.

Estimation of nucleic acid-hydrolysing enzymes. The simple and rapid method described by Kunitz (1946) for the estimation of ribonuclease activity, in which the decrease in extinction at 290 mμ of the ribonucleic acid (RNA) substrate is measured without the necessity of precipitating the undegraded nucleic acid, could not be used in this case because pure substrates were not being used; the estimations were carried out on the crude yeast extracts without the addition of more substrate. This gave a measure of the amount of degradation actually occurring in the extract. The following procedure was adopted for the estimation of this degradation.

The suitably diluted yeast extract in acetate buffer (0·01 M-sodium acetate–0·64 mM-acetic acid), pH 6·0, *I* 0·01, was incubated at 37° in the presence of a few drops of toluene. At intervals samples (1 ml.) were removed and mixed with ethanolic 0·3 M-zinc chloride (0·5 ml.). After 10 min. the solutions were centrifuged at 3000 rev./min. for 3 min. A sample (1 ml.) of the supernatant liquid was removed, diluted five times and the extinction at 260 mμ determined. The zinc chloride–ethanol reagent gave more consistent results than a hydrochloric acid–ethanol reagent and, unlike calcium chloride–ethanol (Jones *et al.* 1957), it could be used in the presence of ammonium sulphate. Determinations were carried out at *I* 0·01, 0·1, 1·0 and 3·0. With the last two the ionic strength was maintained by means of ammonium sulphate. The effect of 0·6 mM-Zn^{2+} ions on the activities at these various ionic strengths was also studied. The results are shown in Fig. 1.

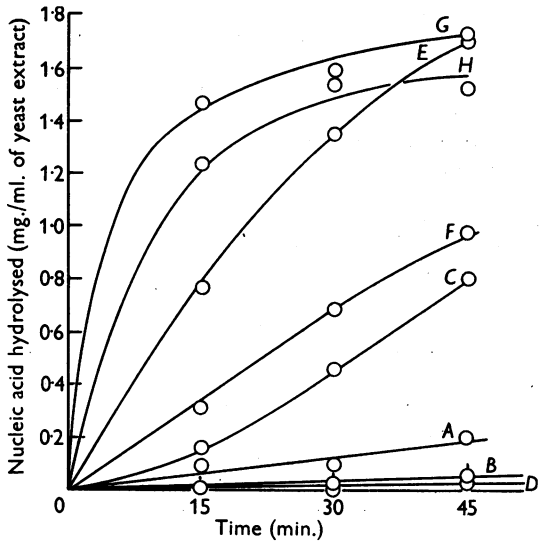

Fig. 1. Enzymic hydrolysis of nucleic acids in extracts of disintegrated yeast at 37°. *A* and *B*, *I* 0·01; *C* and *D*, *I* 0·10; *E* and *F*. *I* 1·0; *G* and *H*, *I* 3·0. *B*, *D*, *F* and *G* contained 0·6 mM-Zn^{2+} ions. The amount of nucleic acid hydrolysed was calculated from the extinction at 260 mμ on the basis that a solution of RNA containing 50 μg./ml. has *E* 1·0. It was found that the small contribution of other ultraviolet-absorbing components could be neglected in this instance.

Preparation of yeast extracts. A suspension of baker's yeast [120 g. of moist pressed yeast (The Distillers Co. Ltd.) equivalent to 30 g. of freeze-dried yeast] in water (250 ml.) was adjusted to pH 7·5 with sodium bicarbonate solution and shaken in a vibratory ball mill (Griffin and George Ltd.) at 0° with an equal volume of Ballotini glass beads (1 mm. diam.). From time to time the pH was readjusted to 7·5 with sodium bicarbonate solution. When almost complete breakage of the cells had been achieved (2½–3 hr.), the liquid was decanted from the glass beads, the latter were washed with water and cell debris was removed from the combined liquid and washings by centrifuging at 20 000 *g*. The supernatant liquid was dialysed against acetate buffer, pH 6·0, *I* 0·01.

Preparation of a standard bentonite suspension. A suspension of bentonite (10 g.; technical grade, British Drug Houses Ltd.) in water (200 ml.) was prepared by thorough stirring and the coarse particles were removed by centrifuging at 2600 rev./min. for 15 min. The fine clay suspension was decanted off and centrifuged at 9000 *g* for 20 min. The cloudy supernatant liquid was discarded and the sediment suspended in acetate buffer, pH 6·0, *I* 0·01. After homogenization in the Ato-Mix blendor (Measuring and Scientific Equipment Ltd.) the suspension was centrifuged at 9000 *g* and the supernatant liquid discarded. This process of suspension in acetate buffer and centrifuging (now at 20 000 *g*) was repeated until the extinction at 260 mμ of the supernatant liquid in a 1 cm. cell had decreased to about 0·7. The bentonite was finally suspended in the acetate buffer (50 ml.).

Adsorption of the nucleic acid-hydrolysing enzymes by bentonite. Samples (10 ml.) of the yeast extract in acetate buffer, pH 6·0, *I* 0·01, were mixed with various volumes of the bentonite suspension. After shaking the suspensions at 0° for 17 hr. the bentonite was removed by centrifuging at 9000 *g*. The enzymic degradation occurring at 37° in the supernatant liquids was determined after increasing the ionic strength to 2·4 by the addition of ammonium sulphate, and the ultraviolet-absorption spectra of the supernatant liquids were also measured. The results are shown in Fig. 2.

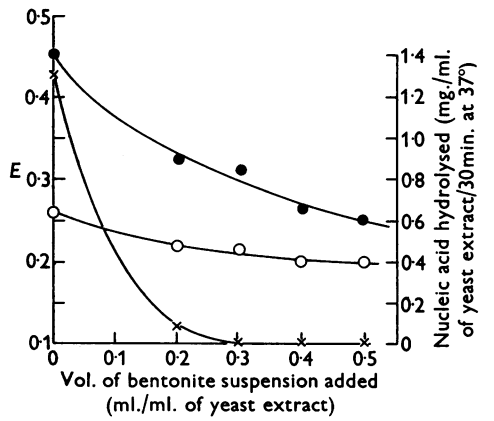

Fig. 2. Effect of bentonite on the ultraviolet absorption and on the activity of nucleic acid-hydrolysing enzymes in an extract of disintegrated baker's yeast in acetate buffer, pH 6·0, *I* 0·01. ●, $E_{220\,m\mu}$; ○, $E_{260\,m\mu}$; ×, enzymic hydrolysis of nucleic acids.

Isolation of ribonucleic acids and ribonucleoproteins

An extract of yeast in acetate buffer, pH 6·0, *I* 0·01, containing 0·6 mM-Zn^{2+} ions (prepared from 225 g. of moist, pressed yeast as previously described except that the yeast was broken in the presence of 0·6 mM-Zn^{2+} ions) was shaken for 18 hr. at 0° with standard bentonite suspension (420 ml.) and the bentonite then removed by centrifuging at 20 000 *g*. After correction for dilution, the extinction of the supernatant liquid at 260 mμ had been reduced 24% by the action of the bentonite. The bentonite-treated solution was adjusted to an ethanol concentration of 20% by the addition of ethanol–acetate buffer, pH 6·0, *I* 0·05 (containing 3 mM-zinc acetate) (4:1) previously cooled to −10°. The zinc concentration was increased to 0·01 M and the temperature maintained at 0°. The material so precipitated was centrifuged off and dissolved in acetate buffer, pH 6·0, *I* 0·01, containing 0·6 mM-Zn^{2+} ions, and dialysed against the same buffer for 18 hr. The zinc and ethanol fractionation was repeated, 96% of the material being precipitated at a Zn^{2+} ion concentration of 0·8 mM in 20% ethanol at pH 6·0 and at 0°. The precipitated nucleoprotein was dissolved in water and a portion of the solution dialysed against distilled water and freeze-dried to give the nucleoprotein YNP [102 mg.; nitrogen, 12·1%, phosphorus, 6·3%; nitrogen/phosphorus (w/w) 1·92; protein, 5·0%]. The remainder of the solution was fractionated with ammonium sulphate, 80% of the material being precipitated at 25% (w/v) of ammonium sulphate. This fractionation was repeated twice. The precipitate was finally dissolved in water and exhaustively dialysed against distilled water. There was, however, still present inorganic material, probably from the bentonite, so the nucleic acid was precipitated with cetyltrimethylammonium bromide (Jones, 1953). The resulting precipitate was dissolved in

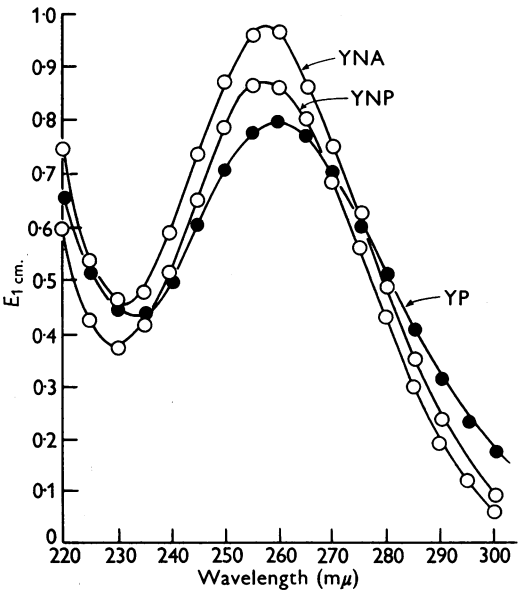

Fig. 3. Ultraviolet-absorption spectra in water of RNA-containing fractions of yeast (50 μg./ml.).

Table 1. *Ribonucleotide content of ribonucleic acid-containing fractions from yeast*

Fraction	Mol. of ribonucleotide per 100 mol. of nucleotides				Recovery of ribonucleotides (% based on phosphorus)
	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	
YNP	26·3	27·5	20·2	25·9	90
YNA	28·3	26·1	21·0	24·6	99
YP	25·7	24·9	22·6	26·8	92

M-sodium chloride, the nucleic acid precipitated by the addition of ethanol, the precipitate was dissolved in water, dialysed and freeze-dried [YNA; 167 mg.; nitrogen, 16·7%; phosphorus, 9·4%; nitrogen/phosphorus (w/w) 1·76; protein, 2·0%]. The combined supernatant liquids from the three ammonium sulphate fractionations were combined, dialysed and freeze-dried to give YP [25 mg.; nitrogen, 16·1%; phosphorus, 6·7%; nitrogen/phosphorus (w/w) 2·4; protein, 15%]. The three fractions contained no deoxyribonucleic acid (Dische, 1930), no ammonia and only traces of Zn^{2+} ions.

An indication that these methods were successful in inhibiting the ribonuclease was that the ammonium sulphate fractionations were reproducible and no nucleic acid was lost when removing the ammonium sulphate by dialysis. The yield of RNA-containing fractions represented, after making allowance for material used for analysis during the fractionation procedures, about 20% of that originally contained in the organisms. The losses were due to incomplete extraction from the organisms and to the fact that, in order to obtain a pure product, nucleic acid-containing fractions were rejected at various stages in the purification.

The three fractions, which had typical ultraviolet-absorption spectra (Fig. 3), were analysed for ribonucleotides by Crosbie, Smellie & Davidson's (1953) method as modified by Jones, Rizvi & Stacey (1958). The results are shown in Table 1.

Electrophoresis. YNP and YNA were subjected to boundary electrophoresis in the Antweiler micro-electrophoresis apparatus. A 2·5% solution of the sample was used in each case. The buffers used were: citrate, pH 3·5, *I* 0·02; phosphate, pH 7·7, *I* 0·1; borate, pH 9, *I* 0·1. The runs were carried out for 15 min. at 80 v. The currents were about 0·54, 1·5 and 2·1 mA for the citrate, phosphate and borate buffers respectively.

Fractions YNP and YNA showed only one peak at pH 7·7 and 9·0. At pH 3·5, YNA showed one rapidly moving component and YNP showed two components, the faster one comprising about 75% of the total.

Amino acid composition of the nucleoprotein YNP. YNP (5 mg.) was treated with crystalline ribonuclease (0·01 mg., free from protease) in acetate buffer, pH 6, *I* 0·01 (5 ml.), at 37° for 18 hr. and the digest was exhaustively dialysed. The nucleic acid content of the non-diffusible fraction was thereby reduced by about 60%. The material remaining inside the bag was hydrolysed by boiling with 6N-hydrochloric acid for 16 hr. The neutralized hydrolysate was subjected to two-dimensional chromatography on Whatman no. 1 paper with methanol–water–pyridine (80:20:4, by vol.) and 2-methylpropan-2-ol–butan-2-one–diethylamine–water (40:40:4:20, by vol.) as the solvents (Redfield, 1953).

RESULTS

Properties of the nucleic acid-hydrolysing enzymes of yeast. The presence of these enzymes in the yeast extracts was indicated when attempts to purify yeast nucleoproteins by ammonium sulphate precipitation at 0° resulted in loss of the majority of the material on subsequent dialysis. A study of the enzymic activity of yeast extracts showed (Fig. 1) that at *I* 0·01 and at 37° some degradation of the nucleic acids was occurring. An increase in ionic strength resulted in increased degradation. This degradation was almost completely inhibited by 0·6 mM-Zn^{2+} ions at *I* 0·01 and 0·1 but only slightly inhibited by this concentration of Zn^{2+} ions in the presence of ammonium sulphate at *I* 1·0 and 3·0. Other experiments were carried out in which higher concentrations of zinc were used, but in no case was complete inhibition of the enzymes achieved at high ionic strengths. Other substances were tried as inhibitors, e.g. *p*-chloromercuribenzoic acid, *o*-iodosobenzoic acid, sodium arsenite and sodium fluoride. These were either without effect or showed insufficient inhibition. Copper sulphate precipitated the yeast nucleoproteins but, upon redissolving them, the hydrolytic enzymes were found to be still present. The degradation of the nucleic acids must have been due mainly to a ribonuclease or a non-specific phosphodiesterase, because there was very little deoxyribonucleic acid present in the yeast extracts and the other enzyme capable of degrading RNA, namely polyribonucleotide phosphorylase (Ochoa, 1957), requires the presence of phosphate. Since the behaviour of this enzyme system shows some similarities to pancreatic ribonuclease the enzyme will be called a ribonuclease, although there is no evidence that its specificity is the same as that of pancreatic ribonuclease.

Preferential adsorption of the yeast ribonuclease. A number of adsorbents were tried; charcoal adsorbed the enzymes but adsorbed RNA and ribonucleoprotein as well, and Zeo-Karb 225 and 226 (cf. Hirs, Moore & Stein, 1953) did not adsorb the enzymes sufficiently strongly. The clay, bentonite, which has been used to purify lysozyme (Alderton, Ward & Feveld, 1945), was able, however, to adsorb the ribonuclease from yeast extracts

(Fig. 2). The extinction of the extracts at 260 mμ had been reduced only 16% by the bentonite treatment, showing that little ribonucleoprotein had been adsorbed. The extinction at 220 mμ was reduced much more than that at 260 mμ. Essentially the same results were obtained by using a yeast extract prepared by disintegrating yeast in 0·6 mM-Zn^{2+} ions so as to inhibit the ribonuclease in the initial stages of any isolation procedure for RNA or ribonucleoprotein. The treatment with bentonite also removed proteolytic enzymes.

Properties of the ribonucleic acid and ribonucleoprotein. The nitrogen and phosphorus analyses and ultraviolet-absorption spectra showed that the fractions isolated were complexes of nucleic acid and protein. The relatively low values for YNP were due to the presence of inorganic material which was removed by subsequent treatment with cetyltrimethylammonium bromide. The negative reactions with the Dische test for 2-deoxy-D-ribose and the identification and estimation of ribonucleotides in the alkaline digests of the fractions showed that the nucleic acid was RNA.

There was a discrepancy between the results for the protein contents determined from nitrogen analyses and those determined by using the Folin–Ciocalteu reagent. This can be explained by the fact that different proteins give different colour intensities with this reagent and therefore the results are not absolute. Multiplication of the results by 1·5 gives good agreement in all three cases with those calculated from nitrogen analyses.

The main difficulty in determining the amino acid composition of the products was their low protein contents. The RNA content could be reduced by 60% by the action of ribonuclease but the remainder seemed resistant to the action of the enzyme. Glutamic acid, aspartic acid, cystine and glycine (possibly from the decomposition of purines) were detected in hydrolysates. Other amino acids were present but they could not be definitely identified owing to disturbance of the chromatograms, probably by the products of hydrolysis of the RNA.

DISCUSSION

The properties of the yeast ribonuclease showed similarities to those of pancreatic ribonuclease in the effect of Zn^{2+} ions and low ionic strength (Zittle, 1946; Davis & Allen, 1955). Zinc ions could be used to inhibit the yeast ribonuclease at I about 0·1 but not at higher ionic strengths. The removal of the ribonuclease activity from the yeast extracts by bentonite was probably due to adsorption of the enzyme. This conclusion appears to be justified in that no enzyme activity could be detected in the bentonite-treated extracts even after the addition of ammonium sulphate and although sufficient substrate was present. Large amounts of protein were adsorbed on the bentonite, and in similar work on the ribonuclease of *Klebsiella aerogenes* (C. R. Bayley & A. S. Jones, unpublished work) the ribonuclease could be eluted off the bentonite. It is well known that certain enzymes are readily adsorbed on clays, e.g. trypsin and chymotrypsin on kaolinite (McLaren, 1954), and lysozyme on bentonite (Alderton *et al.* 1945). It was not surprising therefore that the proteolytic enzymes of yeast were adsorbed also. Thus the consecutive use of Zn^{2+} ions and bentonite adsorption provides for the first time a procedure in which RNA and ribonucleoprotein can be isolated from yeast under very mild conditions of temperature and pH and without enzymic degradation of the RNA.

The ribonucleoprotein that was isolated from the bentonite-treated extracts by zinc and ethanol fractionation was remarkable for its low protein content (5–7%). This may have been due to the bentonite treatment because material isolated in other experiments, without this treatment, contained at a similar stage in the isolation procedure about 60% of protein. At present it is impossible to say whether bentonite was acting as a deproteinizing agent and breaking weak nucleic acid–protein bonds or whether the material with high protein content was an artifact caused by association of protein and nucleoprotein. A deoxyribonucleoprotein containing only 9·6% of protein has been isolated from bovine tubercle bacilli (Tsumita & Chargaff, 1958).

Fractional precipitation of the nucleoprotein with ammonium sulphate did not completely dissociate the protein from the nucleic acid. This indicated the presence of linkages other than salt linkages. The RNA (YNA, 2–3% of protein) was titrated by Dr A. R. Peacocke, who found a small hysteresis loop in the acid region similar to that found with the RNA of *Klebsiella aerogenes* (Cox, Jones, Marsh & Peacocke, 1956; Jones & Peacocke, 1957). This could be attributed to hydrogen bonds but there was no evidence that these bonds linked the nucleic acid to the small amount of protein present in the sample. Strong evidence for the presence of hydrogen bonds in a ribonucleoprotein isolated from *Escherichia coli* has been obtained by Elson (1958).

SUMMARY

1. Nucleic acid-hydrolysing enzymes, which are present in yeast extracts, were activated by increasing ionic strength. They were inhibited by 0·6 mM-Zn^{2+} ions at $I \leqslant 0·1$ but were not completely inhibited by this and higher concentrations of Zn^{2+} ions at higher ionic strengths. Most of the activity was probably due to a ribonuclease.

2. The ribonuclease and also proteolytic enzymes were removed from yeast extracts by adsorption on bentonite.

3. The consecutive use of Zn^{2+} ions and bentonite provided a mild method for the isolation of ribonucleic acids and ribonucleoprotein in which the ribonucleic acids had not been enzymically degraded.

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The Biosynthesis of Phospholipids by Human Blood Cells

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For studies on the phospholipids of human blood cells information is required both on the chemical structures of the individual molecules and on their respective metabolic activities. Although the functions of cellular phospholipids are not well defined, these compounds form an integral part of all cell membranes. Finean (1957) has discussed the structure of nerve myelin, in which the ratio of cholesterol:phospholipid:cerebroside is 2:2:1. Lovelock (1955*a*) showed that phospholipid diffuses readily from human blood cells in considerable quantities when these are washed or treated with alumina. Moreover, a constant phospholipid:cholesterol ratio is necessary for the maintenance of the integrity of the cells (Lovelock, 1955*b*). The differences, if any, between the roles of the different classes of phospholipids, e.g. kephalins, lecithins and sphingomyelins, are still obscure.

It has been shown that ^{14}C-labelled acetate is incorporated into rabbit blood cells *in vivo* (Altman, Whatman & Salomon, 1951) and *in vitro* (Altman, 1953). James, Lovelock & Webb (1957) showed that acetate was incorporated into the fatty acids of human blood cells *in vitro*. The incorporation of [^{32}P]orthophosphate *in vivo* into phospholipids of human blood cells has been studied (Tuttle, Scott & Lawrence, 1939; Erf, Tuttle & Lawrence, 1941; Lawrence, Erf & Tuttle, 1941), but there appears to have been little work on the incorporation *in vitro*.

In the following experiments two approaches to lipid metabolism were combined and the concomitant incorporations of [^{32}P]orthophosphate and [Me-^{14}C]acetate into the phospholipids of human blood cells were studied. The separation of human blood cellular phospholipids into the main classes by chromatography on silicic acid is described. Analyses were made of the fatty acids in each class. Moreover, an attempt was made to measure the metabolic activity of each class both from the point of view of the fatty acid chains and the phosphate groups.

METHODS

Incubation of blood. Freshly drawn whole blood was added to a mixture of acid–citrate–dextrose (0·73 M-trisodium citrate, 0·35 M-citric acid, 0·1 M-glucose; 15 ml./100 ml. of whole blood); [^{32}P]orthophosphate (20–50 μc/ml.); sodium [Me-^{14}C]acetate (3–10 μc/ml., specific activity