THE PREPARATION AND SOME PROPERTIES OF A LOW MOLECULAR WEIGHT RIBONUCLEIC ACID FROM BAKER'S YEAST

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SUMMARY

A low molecular weight RNA has been extracted from whole baker's yeast cells with phenol and compared with yeast S-RNA. Several properties which have been investigated are very similar for both products. Among these properties are the sedimentation constants, the chromatographic behavior on DEAE-cellulose columns, the formation of dinitrophenylhydrazones, the base composition, and the valine and adenylate accepting activity. The extraction procedure is adaptable to large scale preparations.

INTRODUCTION

The interest raised by a ribonucleic acid fraction of low molecular weight[1], able to accept activated amino acids and to transfer them to microsomes, made desirable a search for a readily available source, from which this material could easily be extracted. When living cells are homogenized and the homogenate is fractionated by differential centrifugation, this RNA is normally found in the 100,000 $\times$ g supernatant. Although it has been questioned whether the fraction of RNA able to accept amino acids (transfer RNA)[2] represents all of the supernatant or "soluble" RNA, even after prolonged centrifugation, transfer RNA from mammalian cells has usually been prepared from the 100,000 $\times$ g supernatant fraction. A similar technique has been used by TISSIERES *et al.*[3] for preparing transfer RNA from *E. coli*. While these techniques take advantage of the apparent intracellular localization of the material to be prepared, they unfortunately are long and not readily able to yield large quantities of RNA. PREISS *et al.*[4] have devised a different procedure, based on the low molecular weight of transfer RNA, to separate it from the bulk of *E. coli* RNA, extracted with hot detergent. In previous reports from this laboratory[5,6], it was shown that pressed baker's yeast can yield a fraction of RNA active as an acceptor of both activated amino acids and nucleoside triphosphates (ATP and CTP). The

Abbreviations: RNA, ribonucleic acid; S-RNA, soluble ribonucleic acid; ATP, adenosine triphosphate; CTP, cytidine triphosphate; AMP, adenosine-5'-phosphate; DNPH, 2,4-dinitrophenylhydrazone; DEAE-cellulose, diethylaminoethyl-cellulose.

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present communication is concerned with a brief description of a technique, essentially derived from KIRBY's procedure[7], yielding (from pressed baker's yeast) an RNA fraction of low molecular weight. Properties of this RNA are also compared with those of the soluble RNA obtained after high speed centrifugation of disrupted yeast cells and with the properties of yeast ribosomal RNA. The conclusion is reached that the RNA extracted by this simple procedure is very similar to the S-RNA prepared by the more elaborate centrifugation-precipitation method previously employed.

METHODS

Preparation of the low molecular weight RNA

To 100 g of fresh pressed baker's yeast are added 200 ml of water and 300 ml of phenol–water (90:10, w/w). After 2 h of stirring at room temperature, the mixture is centrifuged and the aqueous layer recovered. The RNA is precipitated with cold ethanol–acetate, according to the method of KIRBY[7]. The precipitate is then dissolved in approx. 40 ml of water at room temperature. If any insoluble precipitate appears on standing at 0° for 10 min, it is removed by centrifugation for 10 min at 25,000 × *g* at 0°. From this point on, all operations are performed at or near 0°. The supernatant is stirred with 1 g of acid washed charcoal (Norite) for 20 min. The charcoal is eliminated by high speed centrifugation and filtration (if necessary) through a rapid filter paper (Whatman No. 41). The charcoal is washed with 5 ml of water. The RNA from combined charcoal wash and supernatant is precipitated with cold ethanol as before. The precipitate is dissolved in approx. 17 ml of water and any insoluble residue removed by centrifugation. The clear solution is extracted twice with 2-methoxyethanol according to the method of KIRBY[7]. The RNA is recovered from the upper layer by adding 0.1 volume of 20% potassium acetate, pH 5.0, and 2.5 volumes of cold ethanol. The precipitate, recovered by centrifugation, is dissolved in the minimum amount of water and dialyzed in the cold against three changes of distilled water (2 l each) over a period of 24 h. The yield is 70–80 mg of RNA.

Preparation of S-RNA and ribosomal RNA

The supernatant fraction of the yeast cells is prepared by rupturing the cells followed by centrifugation, according to CHAO AND SCHACHMAN[8]. At the end of the final 3-h centrifugation at 78,000 × *g*, the upper part of the supernatant is recovered. A loosely packed sediment, appearing on top of the tightly packed ribosome pellet, is left behind. S-RNA is isolated from the fraction by phenol extraction. A charcoal treatment is performed as previously described. For preparing ribosomal RNA, the surface of the ribosome pellet is washed free of poorly sedimented material, using CHAO AND SCHACHMAN's medium[8]. The ribosomes are then suspended in the same medium and treated with 90% phenol, and the RNA recovered, as described previously.

Enzyme preparations

In the case of the Ehrlich ascites enzymes, the S_4 fraction, described by HECHT *et al.*[9], is used.

For the preparation of yeast enzymes, pressed baker's yeast is homogenized with three times its weight of glass beads and 1 volume of CHAO AND SCHACHMAN's medium[8]

using a Virtis 45 homogenizer, cooled with ice, at maximum speed for a total of 15 min. Two drops of octyl alcohol and 50 mg (for 30 g of yeast) of reduced glutathione are added at the start, and the homogenization is carried out in three periods separated by 3-min intervals. Under these conditions the temperature inside the homogenizer does not rise over 14°. From this point on, all operations are performed at or near 0°. One volume of medium is used to transfer the preparation to centrifuge tubes, and the supernatant is recovered after a 5-min centrifugation at 1500 × *g*. The glass beads are washed 3 times with 1 volume of medium, by centrifugation under the same conditions. The combined supernatant fractions are centrifuged at 78,000 × *g* for 3 h. The supernatant, removed from a loosely packed sediment is recovered and processed according to the following procedure:

Immediately after preparation, this supernatant (100 ml), the pH of which is approx. 6.5, is brought to pH 5.0 with 10% acetic acid. About 5 min after the beginning of the operation, the precipitate which forms is collected by centrifuging at 25,000 × *g* for 5 min, and is washed once with 15 ml of 0.1 *M* sodium acetate, pH 5.0. The pellet is then suspended in 0.05 *M* Tris–HCl buffer, pH 7.6, containing 0.005 *M* $MgCl_2$, and the pH is adjusted to 7.6 with 1 *N* KOH. An insoluble residue is eliminated by centrifugation for 10 min at 25,000 × *g*. 1 g of streptomycin sulfate is added with stirring to the opalescent supernatant and the mixture is allowed to stand for 15 min with occasional stirring. The precipitate is eliminated by centrifugation for 10 min at 25,000 × *g*. The resulting supernatant is stirred with 4 g of a mixed bed resin preparation (AG 501-X8)* for 2 min. The resin and a protein precipitate are removed by centrifugation at 1,500 × *g* for 2 min, and are washed twice with 5 ml of water. The pH of the combined supernatants is brought to 7.6 by the addition of 0.05 volume of 1 *M* Tris–HCl buffer, pH 7.6, and the fraction is dialyzed overnight against 0.05 *M* Tris–HCl buffer, pH 7.6 and 0.005 *M* $MgCl_2$. A precipitate forms which is eliminated by centrifugation. The resulting enzyme preparation can be stored at —10°.

Enzyme activity determinations

Incorporation of [^{14}C]valine and [^{14}C]ATP into RNA was carried out according to previously published techniques[5,9]. Incubation mixtures for measuring [^{14}C]valine incorporation contained in a total of 1 ml: 10 μmoles of Mg-ATP, 0.5 μmole CTP, 5 μmoles of phosphopyruvate, 0.01 mg of phosphopyruvate kinase, 0.1 μmole of [^{14}C]valine ($4.5 \cdot 10^6$ counts/min/μmole), 100–200 μg RNA, 50 μmoles Tris–HCl buffer (pH 7.6), and 5 μmoles $MgCl_2$. For measuring [^{14}C]ATP incorporation 1 μmole of [^{14}C]ATP ($1.7 \cdot 10^5$ counts/min/μmole) was used, and the [^{14}C]valine was omitted. Other constituents were the same as used for the valine incorporation experiments. Incubation was carried out at 37°.

Nucleic acid procedures

RNA was determined spectrophotometrically at 260 mμ after 1 h hydrolysis in 1 *N* NaOH at room temperature, as described[9].

The ratios between adenylic acid, guanylic acid, cytidylic acid, uridylic acid and uracil-5-ribosyl phosphate were determined after alkaline hydrolysis according to a modification of the procedure of DAVIS *et al.*[10]. Minor base constituents were isolated and determined according to the method of DUNN[11].

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Amino acids attached to transfer RNA are eliminated by incubation in 0.1 *M* potassium carbonate buffer, pH 10.0, for 50 min at 37°, followed by extensive dialysis of the neutralized solution in the cold.

Reaction of RNA with periodate and 2,4-dinitrophenylhydrazine

The carbon atoms carrying vicinal 2′- and 3′-hydroxyl groups of the terminal ribose residues of transfer RNA, free of attached amino acids, are oxidized with 0.1 *M* sodium periodate in 0.05 *M* sodium acetate buffer, pH 5.0, for 1 h at room temperature in the dark. Most of the excess periodate is precipitated by the addition of 0.1 volume of 2 *M* KCl at 0°. After removal of KIO_4 by centrifugation, the supernatant is dialyzed. The aldehyde groups formed can be reacted with 2,4-dinitrophenylhydrazine under the following conditions: to 4 volumes of oxidized RNA solution, containing up to 5 mg of RNA/ml, are added 1 volume of 0.5 *M* sodium acetate buffer, pH 4.0, 3 volumes of 2-methoxyethanol free of peroxides and 4 volumes of a 1.2 % solution of 2,4-dinitrophenylhydrazine in 2-methoxyethanol. The mixture is incubated at 37° in the dark for 1 h. The excess reagent is eliminated by extraction 5 times with ethyl acetate, after first diluting the incubation mixture with 6 volumes of water. The 2,4-dinitrophenylhydrazone derivative of RNA (DNPH-RNA) is recovered by alcoholic precipitation. The extent of the reaction can be followed by measuring the extinction at 360 mμ, assuming a molecular extinction coefficient of 21,000 for the DNPH radical[12].

RESULTS

Sedimentation constants

The values of the sedimentation constants, determined in 0.01 *M* phosphate buffer, pH 7.0, at 59,780 rev./min in the analytical rotor of the Spinco model E ultracentrifuge, at a concentration of 0.005 % of RNA, are 4.3, 4.4 and 6.1 S respectively, for the RNA extracted by the phenol procedure from whole cells, the S-RNA, and the ribosomal RNA. The sedimentation patterns of the first two RNA samples do not give any indication of heterogeneity.

Solubility in concentrated salt solutions

While the RNA extracted from the ribosomes is completely insoluble in 1 *M* NaCl and in 65 % saturated ammonium sulfate[2], both the S-RNA and the RNA extracted from whole cells by the phenol procedure are completely soluble in 1 *M* NaCl. In 65 % saturated ammonium sulfate, the latter RNA is only partially soluble, but there is no difference in valine-accepting activity between the products recovered from the precipitate and from the supernatant.

Chromatographic behavior

The elution patterns obtained for the three samples of RNA by gradient elution chromatography on DEAE-cellulose columns are presented in Fig. 1. The RNA extracted from whole cells and the S-RNA both are eluted between 0.40 *M* NaCl and 0.56 *M* NaCl. The recovery is 94–97 % of the RNA originally put on the column. In the case of the ribosomal RNA, only approx. 7 % of the initial amount is eluted in this range of concentrations. The rest can be eluted with 0.1 *N* NaOH.

Nucleotide composition

The amounts of adenylic acid, guanylic acid, cytidylic acid, uridylic acid, uracil-5-ribose phosphate, thymine ribotide and 6-methylaminopurine ribotide, expressed relative to adenylic acid are given in Table I. The composition of the RNA extracted from whole cells is essentially identical to that of the S-RNA, within the limits of the precision of the method used. This composition is, however, very different from that of the ribosomal RNA.

Reaction with periodate and 2,4-dinitrophenylhydrazine

Evidence has been presented for the linkage of the amino acids to the 2'- or 3'-hydroxyl group of the ribose of the terminal adenine nucleotide of S-RNA[4,5,13]. When the amino acids have been removed from S-RNA by treatment with alkali, the terminal ribose of S-RNA contains hydroxyl groups on the 2'- and 3'-position of the ribose. These are available for oxidation by periodate to form a dialdehyde on the terminal ribose of the RNA chain.

It had previously been observed that the periodate reaction with S-RNA was blocked by the presence of amino acid in 3'- or 2'-ester linkage on the terminal ribose[4,5,13], and conversely that the attachment of amino acid to the terminal ribose of S-RNA was prevented by previous periodate oxidation of the S-RNA[4]. These two pieces of evidence point to the adjacent 2'- and 3'-hydroxyl groups of the terminal ribose unit of the S-RNA as being the site of periodate oxidation.

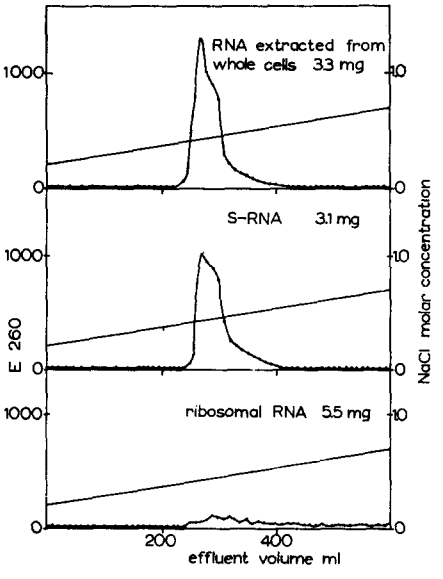

Fig. 1. Chromatography of RNA samples on DEAE-cellulose columns. Columns: 1.2 × 12 cm. A linear gradient of NaCl concentration is established using a mixer with 500 ml of 0.02 *M* potassium phosphate buffer, pH 7.7, containing 0.2 *M* NaCl and a reservoir with 500 ml of 0.2 *M* potassium phosphate buffer, pH 5.5, containing 1.2 *M* NaCl.

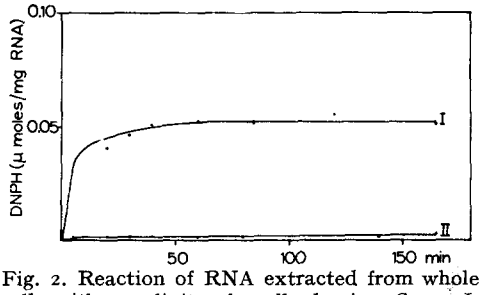

Fig. 2. Reaction of RNA extracted from whole cells with 2,4-dinitrophenylhydrazine. Curve I, RNA freed of attached amino acids by incubation at pH 10 and oxidized with periodate; curve II, RNA freed of attached amino acids.

When transfer RNA free of attached amino acids and oxidized with periodate is treated with 2,4-dinitrophenylhydrazine, a hydrazone is rapidly formed, apparently with these aldehyde groups of the terminal ribose. In the absence of periodate oxidation, there is essentially no reaction of S-RNA with dinitrophenylhydrazine during an incubation period of 2 h. The time course of the reaction is shown in Fig. 2.

It is essential that the nucleic acid samples be treated with 2-methoxyethanol

TABLE I

NUCLEOTIDE COMPOSITION OF RNA SAMPLES

	Molar ratio; adenylic acid = 10.0					
	Guanylic acid	*Cytidylic acid*	*Uridylic acid*	*Uracil-5-ribosyl phosphate*	*Thymine ribotide*	*6-methylamino-purineribotide*
RNA extracted from whole cells	16.1	15.9	10.2	2.2	0.32	0.19
S-RNA	15.8	15.3	10.8	2.1	0.29	0.17
Ribosomal RNA	10.7	7.7	10.1	0.3	< 0.04	< 0.04

prior to oxidation by periodate, since contamination by polysaccharide yields spuriously high values. Under these conditions Table II indicates that the attachment of DNPH to RNA was of the same order of magnitude in samples of RNA obtained by phenol extraction of whole cells and those of S-RNA obtained after differential centrifugation while the attachment of DNPH to ribosomal RNA was only 0.2 as much. These figures are not changed if the RNA samples are incubated with prostatic phosphatase before periodate oxidation. It is therefore unlikely that a phosphate monoester is present at the 2′- or 3′-position on this terminal ribosyl unit of the RNA.

TABLE II

REACTION OF PERIODATE OXIDIZED RNA WITH 2,4-DINITROPHENYLHYDRAZINE

	μmoles DNPH/mg RNA
Soluble RNA A	0.0663
Soluble RNA B	0.0614
RNA-phenol extract of whole cell III	0.0543
RNA-phenol extract of whole cell IV	0.0575
RNA-phenol extract of whole cell V	0.0875
Ribosome RNA B	0.0091

Labeling of RNA using [^{14}C]valine and [^{14}C]ATP

It had previously been shown by this laboratory[5,9] that S-RNA from liver or ascites cells was able to incorporate [^{14}C]AMP into the terminal position of the RNA chain, using [^{14}C]ATP as precursor and the appropriate enzymes. Similar samples of RNA are able to bind amino acid to the terminal adenine moiety of the RNA molecule. Table II indicates that using crude yeast enzyme, yeast RNA prepared by phenol extraction of the intact cell behaves like soluble RNA from yeast, in contrast to yeast ribosomal RNA which incorporates these compounds very poorly. These values in the yeast RNA samples are of the same order of magnitude as the incorporation in S-RNA from rat liver or ascites cells[5]. It is also noteworthy that, in agreement with previous findings[5], the incorporation of the mononucleotide precursor into RNA is 10–20-fold greater than that of a single amino acid precursor. This situation is consistent with the presence of a separate transfer RNA molecule for each amino acid. The slight incorporation observed in the ribosomal RNA may be explained as a slight contamination with S-RNA. This is in agreement with the chromatographic pattern observed in Fig. 1.

Table III also indicates that S-RNA and the RNA prepared by phenol extraction

TABLE III

COMPARISON OF LABELING OF VARIOUS TYPES OF YEAST RNA BY YEAST ENZYMES AND ASCITES ENZYMES

	mμmoles incorporated/mg RNA											
	[^{14}C]valine						*[^{14}C]ATP*					
	Yeast enzyme		*Ascites enzyme*		*Both*		*Yeast enzyme*		*Ascites enzyme*		*Both*	
Time (min)	*30*	*60*	*30*	*60*	*30*	*60*	*30*	*60*	*30*	*60*	*30*	*60*
Soluble RNA I	1.01	1.26	0.11	0.0	0.67	0.08	—	11.55	—	0.74	7.00	0.0
Soluble RNA II	0.80	0.80	0.07	0.0	0.37	—	—	8.48	—	0.34	3.25	0.0
RNA-phenol extract of whole cell I	1.07	1.29	0.94	1.12	1.30	1.18	—	21.10	—	20.70	19.15	22.05
RNA-phenol extract of whole cell II	1.43	1.69	1.48	1.45	1.36	1.47	—	25.70	—	25.15	26.80	27.80
Ribosome RNA	0.07	0.09	0.004	0.001	0.05	0.01	—	1.76	—	0.18	0.99	0.0

from the whole cells do not behave in the same way when the ascites enzyme is used. This enzyme is able to promote an incorporation of both [^{14}C]AMP and [^{14}C]valine into the RNA prepared from whole cells only. No incorporation occurs when S-RNA is used as an acceptor. This finding suggests that the S-RNA isolated after centrifugation of the yeast homogenate has lost some of its integrity, possibly due to an enzymic degradation during the lengthy isolation procedure. Apparently the crude yeast enzyme is able to restore this, while the ascites enzyme is not. The parallelism between [^{14}C]AMP and [^{14}C]valine incorporation also suggests that in agreement with previous findings using ascites S-RNA[5], when the RNA sample is unable to receive the terminal adenine, it is also unable to bind amino acid.

DISCUSSION

The properties of the RNA extracted from whole yeast cells and those of yeast S-RNA have been found to be similar in a number of respects. It is likely that, under the extraction conditions used, the cell wall is permeable only to small molecules and does not allow the extraction of ribosomes or ribosomal RNA. The yield of RNA obtained by our procedure (0.07–0.08 %) is compatible with the amount of S-RNA present in the original yeast cake (0.3 %)[8]. It has been shown that the [^{14}C]valine attached to the RNA extracted from whole yeast cells can be transferred to rat liver microsomes under suitable conditions[6]. Whether our RNA preparations are made only of transfer RNA remains to be determined by further work. We cannot at the present time exclude the possibility that our preparations contain a mixture of transfer RNA and of RNA molecules of similar molecular weight but performing a different function or originating from a partial degradation of ribosomal RNA.

We have already mentioned the similarity in activity between S-RNA from yeast and S-RNA from rat liver. If we compare our data on the base composition with those given by MAGASANIK[14] for rat liver S-RNA, a close similarity is again apparent. Comparable compositions have also been found for the S-RNA from *E. coli*[3], from Ehrlich ascites tumor cells and from guinea pig liver (*cf.* ref. 15). OTAKA *et al.*[16,17] have already described a preparation of yeast S-RNA. While their data on the

nucleotide composition are roughly similar to ours, differences do exist on the guanylic acid and cytidylic acid contents. These differences could be due to the differences in the strains of yeast used. Our data do not permit an accurate determination of the molecular weight, but they would be in agreement with a value in the range of 20,000–30,000, which is comparable with the value already found by others for various S-RNA preparations[3,9,11], but definitely smaller than the value of 50,000 reported by PREISS *et al.*[4] for *E. coli* S-RNA.

The fact that the incorporation of [^{14}C]valine into the samples of S-RNA prepared by us is dependent on the incorporation of AMP, indicates that the molecules are not only free of amino acids but even free of terminal AMP residues and therefore are non-functional as isolated. We cannot state at the present time whether this is a consequence of a partial degradation occurring in the course of the preparation or whether this is due to the low levels of the nucleotide and amino acid pools in the resting cells we are using for our preparations.

The chief virtue of the present method is that an RNA, capable of accepting and transferring amino acids in an *in vitro* amino acid incorporation system, may be readily prepared from commercial baker's yeast in large quantities.

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REFERENCES

1 M. B. HOAGLAND, M. L. STEPHENSON, J. F. SCOTT, L. I. HECHT AND P. C. ZAMECNIK, *J. Biol. Chem.*, 231 (1958) 241.
2 K. C. SMITH, E. CORDES AND R. S. SCHWEET, *Biochim. Biophys. Acta*, 33 (1959) 286.
3 A. TISSIERES AND J. D. WATSON, *Nature*, 182 (1958) 778.
4 J. PREISS, P. BERG, E. J. OFENGAND, F. H. BERGMANN AND M. DIECKMANN, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 319.
5 L. I. HECHT, M. L. STEPHENSON AND P. C. ZAMECNIK, *Proc. Natl. Acad. Sci., U.S.*, 45 (1959) 505.
6 P. C. ZAMECNIK, *Harvey Lectures*, Series 54, 1958–1959, in the press.
7 K. S. KIRBY, *Biochem. J.*, 64 (1956) 405.
8 F. C. CHAO AND H. K. SCHACHMAN, *Arch. Biochem. Biophys.*, 61 (1956) 220.
9 L. I. HECHT, P. C. ZAMECNIK, M. L. STEPHENSON AND J. F. SCOTT, *J. Biol. Chem.*, 233 (1958) 954.
10 F. F. DAVIS, A. F. CARLUCCI AND I. F. ROUBEIN, *J. Biol. Chem.*, 234 (1959) 1525.
11 D. B. DUNN, *Biochim. Biophys. Acta*, 34 (1959) 286.
12 J. D. ROBERTS AND C. GREEN, *J. Am. Chem. Soc.*, 68 (1946) 214.
13 H. G. ZACHAU, G. ACS AND F. LIPMANN, *Proc. Natl. Acad. Sci., U.S.*, 44 (1958) 885.
14 B. MAGASANIK, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. I, Academic Press Inc., New York, 1955, p. 373.
15 M. B. HOAGLAND, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. III, Academic Press Inc., New York, in the press.
16 E. OTAKA, Y. HOTTA AND S. OSAWA, *Biochim. Biophys. Acta*, 35 (1959) 266.
17 S. OSAWA AND E. OTAKA, *Biochim. Biophys. Acta*, 36 (1959) 549.