POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

Vol. 23 No. 1

QUARTERLY

ABPLAF 23 (1) 1-84 (1976)

WARSZAWA 1976

PWN POLISH SCIENTIFIC PUBLISHERS

The next issue of Acta Biochimica Polonica, no. 2 - 3, vol. 23 (1976) will be dedicated to the memory of the late Professor Włodzimierz Mozołowski.

OGŁOSZENIE

Komitet Biochemii i Biofizyki PAN ogłasza otwarty konkurs na podjęcie w placówkach krajowych badań w trzech szczególnie ważnych kierunkach biochemii i biologii molekularnej:

1. Wirusologia molekularna
2. Mechanizmy przekazywania sygnałów biologicznych
3. Molekularne podstawy rozwoju biologicznych układów regulatorowych.

Prace te będą finansowane przez Komitet Biochemii i Biofizyki PAN. Zgłoszenie propozycji naukowo-badawczych na lata 1976 - 1980 wraz z podaniem koncepcji planu badań i możliwości jego realizacji proszę uprzejmie kierować na adres:

Sekretarz Komitetu Biochemii i Biofizyki PAN
Prof. dr M. Bagdasarian

Instytut Biochemii i Biofizyki PAN
ul. Rakowiecka 36
02-532 Warszawa

POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

Vol. 23 No. 1

CONTENTS

QUARTERLY WARSZAWA 1976

PWN POLISH SCIENTIFIC PUBLISHERS

ADDRESS

ul. Banacha 1, 02-097 Warszawa
(Zakład Biochemii A.M.)
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakład 934+116 egz. Ark. wyd. 8,25, ark. druk. 5,75+0,25 wkł.
+0,125 kr. Papier druk. sat. kl. III, 80 g. 70×100
Oddano do składania 2.XII.75 r. Podpisano do druku 12.V.76 r.
Druk ukończono w maju 1976 r.
Zam 852/75 J-101 Cena zł 25,—

Warszawska Drukarnia Naukowa, Warszawa, Śniadeckich 8

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 1

JACEK AUGUSTYNIAK, URSZULA KARWOWSKA, ANNA GOŹDZICKA, DAMIAN LABUDA and EWA ZARADNIAK

LARGE-SCALE ISOLATION OF tRNA FROM BARLEY EMBRYOS*

Institute of Biochemistry of Poznań Universities, ul. Fredry 10, 61-701 Poznań, Poland

1. Large-scale isolation of tRNA from barley embryos is described, involving: phenol extraction, RNA deproteinization with the chloroform - isoamyl alcohol mixture, batch sorption on DEAE-cellulose, NaCl gradient elution of tRNA from DEAE-cellulose, and deaminoacylation of tRNA in the presence of bentonite. The procedure yielded tRNA free of protein and RNase activity.

2. The amino acid acceptor activity of the crude barley tRNA, its melting profiles and chromatographic patterns on Sephadex G-100 and BD-cellulose were similar to those of tRNA from other sources.

Transfer ribonucleic acids of plant origin are much less known than those isolated from microbial material or from animal tissues. Among more than 70 so far sequenced tRNA structures, there is only one plant tRNA (Dudock *et al.*, 1969). This seems to be due not only to the obvious choice of simpler systems but also to the fact that isolation of tRNA from cells of higher plants is more difficult than from those of yeasts, bacteria or mammalian tissues.

On the other hand, availability of plant material at any desired developmental stage makes plants particularly suitable models for studying the role of tRNA during cell differentiation or ageing of tissues (Shugart, 1972) or for investigation of tRNA regulatory functions (Littauer & Inouye, 1973), including correlation between plant hormones and the isoaccepting tRNA. Isolation of tRNA on a rather large scale is a prerequisite in these studies. The isolation procedure is, however, complicated by large differences in chemical composition of various plants, high variability of plant material and rather high content of ribonucleases.

The present paper deals with the procedure for isolation of tRNA from barley embryos. Barley is comparatively rich in ribonucleases (Félix & Chambon, 1974) and therefore special care was taken to obtain tRNA devoid of endogenous nucleolytic activity.

* This work was supported by the Polish Academy of Sciences within the project 09.3.1.

EXPERIMENTAL

Materials

Barley (*Hordeum vulgare*) embryos were obtained from commercial bran (Oborniki Mill, Poland), sieved to remove grain covers.

DEAE- and BD-cellulose were from Serva (Heidelberg, G.F.R.).

[^{3}H] and [^{14}C]-L-amino acids were from the Radiochemical Centre (Amersham, Bucks., England), Sephadex G-100 and G-200 from Pharmacia (Uppsala, Sweden), bentonite from Sigma (St. Louis, Mo., U.S.A.). Phenol was freshly glass-distilled from the technical-grade reagent. Acrylamide and *N,N'*-methylenebisacrylamide (B.D.H., Poole, Dorset, England) were crystallized from chloroform and acetone, respectively. Other chemicals were reagent grade products from Polskie Odczynniki Chemiczne (Gliwice, Poland).

Methods

Analytical. Electrophoresis on polyacrylamide gel (5 or 7.5%) was performed according to Loening (1967). RNA concentration was determined by measuring absorbance at 260 nm in 1-cm cuvettes; it was assumed that 1 mg of RNA is equivalent to 20 A_{260} units. Protein was determined by the method of Lowry *et al.* (1951), and ribonuclease activity as described by McDonald (1955). Polysaccharide content was calculated by subtracting RNA and protein contents from the total dry weight of the tRNA preparations. Melting curves of tRNA were measured with a Specord UV-Vis spectrophotometer (Carl Zeiss, Jena), equipped with thermostatically controlled cuvette blocks and a calibrated thermoresistor. Transfer RNA, extensively dialysed against 10 mM-$MgCl_2$ and 50 mM-NaCl was placed both in the "reference" and "measuring" cuvettes. Only the latter was heated (at a rate of about 2°/min) with continuous stirring to avoid formation of vapour bubbles. The results, expressed as percentage of hyperchromicity, were corrected for thermal expansion of the solution and for any temperature-dependent changes in absorbance of the solvent.

Aminoacylation and preparation of aminoacyl-tRNA synthetases. The acceptor activity of tRNA was estimated by the filter-paper disc technique as described by Mans & Novelli (1961), and the radioactivity was measured in toluene-based scintillation liquid with Packard Tri-Carb Model 2425 scintillation spectrometer.

Crude aminoacyl-tRNA synthetase was prepared as follows: ground barley grains (100 g) were extracted in a cold-room for 3 h with 300 ml of 0.01 M-potassium-phosphate buffer, pH 7.2, containing 2 mM-$MgCl_2$, 5 mM-2-mercaptoethanol and 10% (v/v) of glycerol. The extract was filtered through gauze and centrifuged for 10 min at 15 000 *g*. To the supernatant, ammonium sulphate was added to 0.7 saturation and the precipitate collected by centrifugation was dissolved in a small volume of 0.01 M-phosphate buffer, pH 7.2, and filtered through Sephadex G-200 column (5 × 34 cm) equilibrated with the same buffer. Fractions emerging at 1.3 -

1.5 V_e/V_0 had the highest aminoacyl-tRNA synthetase activity and were used as the crude enzyme preparation.

Crude yeast aminoacyl-tRNA synthetase, used for localization of specific tRNAs in the effluents from the Sephadex column, was prepared as described previously (Labuda *et al.*, 1974).

Aminoacylation of tRNA was measured in the mixture containing in a final volume of 100 µl: 10 µmoles of Tris-HCl buffer (pH 7.5 for valine, methionine and arginine incorporation, pH 8.0 for leucine and isoleucine, pH 8.5 for phenylalanine); 0.4 µmole of $MgCl_2$; 0.4 µmole of ATP; 3 - 5 nmoles of radioactive amino acid; 0.5 - 3.0 units (A_{260}) of tRNA, and 100 - 200 µg of crude barley synthetase (or about 10 µg of crude yeast enzyme).

Isolation of tRNA

The procedure consisted of the following five steps:

Step 1. Barley embryos (2.5 kg) were extracted with a mixture of 8 litres of freshly distilled phenol saturated with water and 8 litres of the aqueous solution containing 10 mM-$MgCl_2$, 2 mM-EDTA, 3 mM-2-mercaptoethanol, 0.1% (w/v) of sodium dodecyl sulphate and 1% (w/v) of bentonite. The suspension was vigorously stirred for 2 h and centrifuged at 6000 *g* for 20 min. The cell debris was removed, the aqueous phase was stored, the phenol phase was re-extracted with 4 litres of the above mixture for 30 min, and the aqueous washings separated on centrifugation from the phenol phase were pooled with the main extract. All the operations were carried out in a cold-room; separation of phases was performed in a refrigerated preparative centrifuge using 1-litre glass tubes.

Step 2. Bentonite (100 g) and solid NaCl (27 moles) were added to the pooled extract (10 litres) from step 1. The suspension was vigorously stirred for 1 h at room temperature with 0.5 vol. of phenol saturated with water, and centrifuged for 20 min at 6000 *g*. The upper phenol phase was removed by suction and the aqueous phase was extracted three times with 0.3 vol. of a chloroform - isoamyl alcohol mixture (24:1). After each extraction the lower phase and the opaque interphase were removed by centrifugation.

Step 3. The extract from step 2 (about 8 litres) was diluted about tenfold with cold 3 mM-2-mercaptoethanol to lower NaCl concentration to 0.3 M (the concentration of NaCl was determined previously by Cl titration with $AgNO_3$ in the presence of Na_2CrO_4). To the diluted extract, 2 ml of settled DEAE-cellulose, equilibrated with 0.3 M-NaCl, was added per each 100 A_{260} RNA units, and the suspension obtained was left, with occasional stirring, for 30 min in a cold-room. The suspension was filtered under suction through a large sintered-glass funnel (G-2 or G-3), and the collected DEAE-cellulose was washed with 0.3 M-NaCl solution containing 1 mM-EDTA, 2 mM-$MgCl_2$ and 3 mM-2-mercaptoethanol (about 8 litres) until A_{260} of the washings did not exceed 0.8. Nucleic acids were then eluted from DEAE-cellulose with about 2 litres of 1.0 M-NaCl solution containing 1 mM-

EDTA, 2 mM-$MgCl_2$ and 3 mM-2-mercaptoethanol, precipitated with 2.5 volumes of cold (−18°C) ethanol and left for 8 h at −18°C.

Step 4. The precipitated RNA was collected by centrifugation, washed with cold ethanol and, after removal of the excess of organic solvent with tightly rolled filter paper, it was dissolved in 200 ml of 10 mM-acetate buffer, pH 5.0, containing 0.3 M-NaCl, 10 mM-$MgCl_2$, 1 mM-EDTA and 3 mM-2-mercaptoethanol. The solution was cleared by centrifugation for 15 min at 15 000 *g* and applied on DEAE-cellulose column (2.6 × 35 cm) equilibrated with the same buffer. The column was washed with this buffer until A_{260} of the effluent, monitored with Uvicord II LKB, dropped down to the level of the equilibrating buffer. Then tRNA was eluted with a linear NaCl gradient (0.3 - 1.0 M) in the acetate equilibrating buffer (total volume 2000 ml). Fractions emerging from the column between 0.5 and 0.8 M-NaCl were collected and tRNA was precipitated with 2.5 volumes of cold ethanol.

Step 5. Transfer RNA from step 4 was pelleted by centrifugation, dissolved in 200 ml of 0.5 M-Tris-HCl buffer, pH 9.0, containing 10 mM-$MgCl_2$, 2 mM-EDTA and 3 mM-2-mercaptoethanol, and incubated in the presence of 1% (w/v) bentonite for 45 min at 37°C. The incubation mixture was cooled, centrifuged to remove bentonite, and mixed with 2.5 volumes of cold ethanol. The precipitated crude tRNA was collected by centrifugation, washed with ethanol and peroxide-free ethyl ether, and dried in a desiccator under reduced pressure.

RESULTS AND DISCUSSION

The described procedure gave reproducible results in the yield of crude tRNA and in its characteristics. The procedure includes several steps reported previously by others. Phenol extraction of tissues, originally introduced by Kirby (1956), now became a standard procedure. The low salt concentration used at the first step of our technique was introduced to minimize DNA extraction in accordance with the suggestions of Stansly & Seese (1965). The extraction time (2 h) applied at step 1 was a compromise between the desired high yield of RNA and the necessity of shortening the entire procedure.

In accordance with the observations of Robins & McNutt (1974) we found that even repeated phenol extraction did not remove all of the RNase activity from the aqueous phase during RNA isolation, neither was RNase completely removed by the addition of bentonite. Probably the enzyme was "stabilized" in the barley embryos extract not only by RNA (Robins & McNutt, 1974) but also by some other water-soluble substances extracted from the embryos.

Enriching of the aqueous phase with NaCl at step 2 of the procedure was taken from Dudock *et al.* (1969). The high salt concentration lowered solubility of rRNA and also facilitated separation of phases.

The yield of u.v.-absorbing material (A_{260}) at step 2 of the isolation procedure was about 150 000 A_{260} units. Most of this material was not RNA since its u.v. spectrum (Fig. 1) had the maximum at about 270 nm. During batch sorption on

DEAE-cellulose at step 3, only about 20 000 A_{260} units were adsorbed. The non-adsorbed material contained no RNA since: (a) its u.v. spectrum (Fig. 1) showed maximum at a longer wavelength; (b) sorption could not be achieved in the presence of 0.3 M-NaCl even when a large excess of the exchanger was used; (c) purified tRNA, mixed with the non-adsorbed fraction, was quantitatively recovered when 1 ml of DEAE-cellulose was used per each 200 A_{260} units of this mixture. Out of the 20 000 A_{260} units adsorbed on DEAE-cellulose, 15 000 was eluted with 1 M-NaCl. The u.v. spectrum of this fraction (Fig. 1) indicated substantial purification of RNA achieved at this step. The preparation obtained showed, however, ribonuclease activity and still contained about 3% of protein and probably some carbohydrates (1 mg of dry preparation at this purification step yielded about 14 A_{260} units). When, however, instead of adsorption on DEAE-cellulose, RNA was precipitated with ethanol, the preparation contained still more polysaccharides.

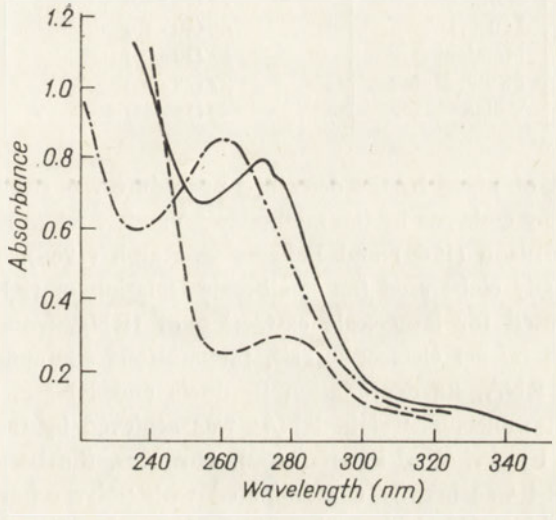


Fig. 1. Ultraviolet absorption spectra of the aqueous phase after purification step 2 (——); the non-adsorbed fraction (— — —); and RNA eluted from DEAE-cellulose with 1 M-NaCl (purification step 3) (—·—·—).

Gel electrophoresis revealed also the presence of small amounts of RNA having mol. wt. higher than 5S RNA. These impurities were subsequently removed at step 4: about 1000 A_{260} units, out of 15 000 units applied on the DEAE-cellulose column, were eluted before the 4S RNA peak, 10 000 were recovered as the main tRNA fraction, and about 4000 units were strongly held by the exchanger. Step 5 was introduced in the purification procedure to deaminoacylate tRNA; bentonite was added to protect tRNA from any incidentally introduced RNases.

The final crude tRNA preparation contained neither protein detectable by the Lowry method, nor RNase activity. Polyacrylamide-gel electrophoresis showed the presence of 5S (about 15%) and 4S RNA (about 85%). The preparation contained,

however, some yellowish pigment. One milligram of this preparation corresponded to 18 A_{260} units. The aminoacid acceptor activity towards 6 amino acids (Table 1) was similar to that of tRNA isolated from other sources (Legocki *et al.*, 1967; Vanderhoef & Key, 1970; Guderian *et al.*, 1972; Pearson *et al.*, 1973).

Table 1

Aminoacid acceptor activity of crude barley tRNA

The results are mean values obtained with at least 5 batches of crude tRNA; limit values are given in parentheses.

Amino acid	Acceptor activity (pmoles of amino acid per 1 A_{260} unit of tRNA)
Arginine	28 (27 - 35)
Isoleucine	35 (28 - 40)
Leucine	46 (40 - 50)
Methionine	32 (30 - 35)
Phenylalanine	27 (25 - 30)
Valine	40 (35 - 45)

Transfer RNA obtained by the described procedure was compared with tRNA isolated from barley embryos by the methods of Vanderhoef & Key (1970), Zubay (1962), Avital & Elson (1969) and Bellamy & Ralph (1968). The Vanderhoef's procedure, originally elaborated for small-scale isolation of tRNA from soybean seedlings, when used for large-scale extraction of tRNA from barley embryos, yielded, as shown by gel electrophoresis, preparations contaminated with high-molecular-weight RNA, its degradation products and RNase. Better separation of low- from high- molecular-weight RNAs was achieved by the use of 2 M-LiCl as recommended by Avital & Elson (1969). However, the barley RNA fraction soluble in 2 M-LiCl was heavily contaminated with polysaccharides which precipitated together with tRNA during subsequent salt fractionation procedure. In consequence, the crude preparation of barley tRNA obtained by this technique contained only 8 - 10 A_{260} units/mg of dry weight. A similarly high content of polysaccharides was found in the barley tRNA prepared by the procedure of Bellamy & Ralph (1968); in addition, the tRNA obtained by this method contained also high-molecular-weight RNAs. The isopropanol fractionation procedure of Zubay (1962), successfully applied by Robison & Zimmerman (1970) in preparation of tRNA from bovine liver, was found to be effective in removing most of the polysaccharides from barley extract. However, tRNA was precipitated together with polysaccharides and the yield of tRNA was diminished by a half as compared with the procedure recommended by us.

For more detailed characterization of crude barley tRNA, the preparation was chromatographed on BD-cellulose column according to Gillam *et al.* (1967). Crude tRNA (15 000 A_{260} units) was applied to a column (2.5 × 130 cm) equilibrated with 0.35 M-NaCl in 0.05 M-sodium-acetate buffer, pH 5.0, containing 10 mM-$MgCl_2$,

2 mM-EDTA, 2 mM-2-mercaptoethanol and 0.01% NaN_3. The successive specific tRNAs were eluted with the NaCl gradient (0.35 - 1.5 M) in the same buffer, generated with the use of Ultrograd (LKB). When absorbance of the effluent dropped down below 0.3, the column was washed with 1.5 M-NaCl in the same buffer containing 10% (v/v) of ethanol. For localization of specific tRNAs in the effluent from BD-cellulose, 50 µl aliquots were taken from every second tube and the aminoacid acceptor activity was measured as described in Methods. Since aminoacylation depended on NaCl concentration, the radioactivity counts were used only for localization of individual tRNAs and not for calculation of their absolute aminoacid acceptor activity. The chromatographic pattern of barley tRNA shown in Fig. 2 is closely similar to the patterns obtained for tRNAs isolated from other sources (Gillam *et al.*, 1967; Dudock *et al.*, 1969; Yoshikami & Keller, 1971; White & Tener, 1973; and review by Kothari & Taylor, 1972).

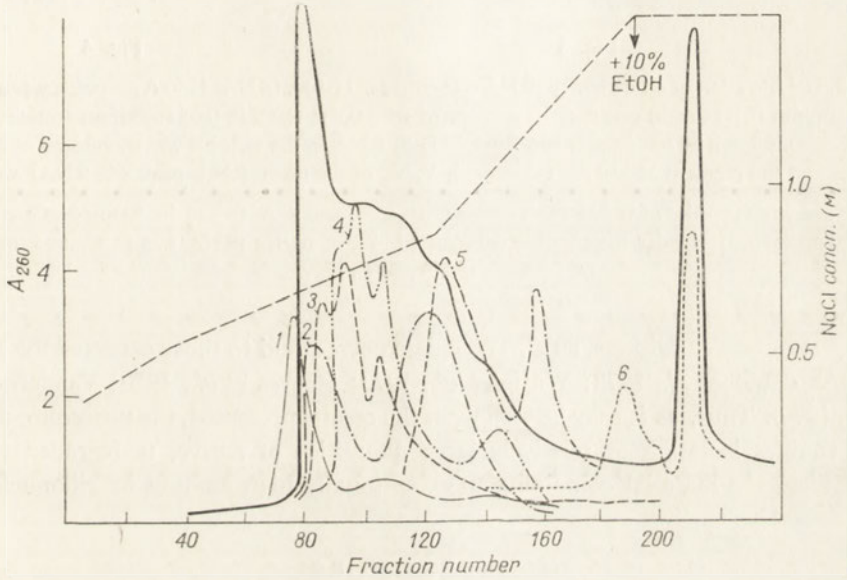


Fig. 2. BD-cellulose chromatography of the purified barley tRNA preparations. Fractions of 10 ml were collected and analysed for the acceptor activity towards: *1*, Met; *2*, Ile; *3*, Val; *4*, Leu; *5*, Arg; *6*, Phe; ——, A_{260}. The radioactivity counts were used only for localization of tRNAs and not for quantitative characterization.

The yellow pigment and 5S RNA could be removed from the crude barley preparation by gel filtration on Sephadex G-100 column equilibrated with 0.5 M-NaCl in 0.05 M-sodium-acetate buffer, pH 4.7, containing 10 mM-$MgCl_2$ and 2 mM-EDTA (Fig. 3).

The melting temp. of barley tRNA purified by Sephadex G-100 chromatography were read from maxima of differential curves ($\Delta A/1°$ plotted *versus* temperature) derived from melting profiles (Fig. 4). Barley tRNA showed 28% of hyperchromicity in 10 mM-$MgCl_2$; its T_m was 81°C, while that of yeast tRNA, measured

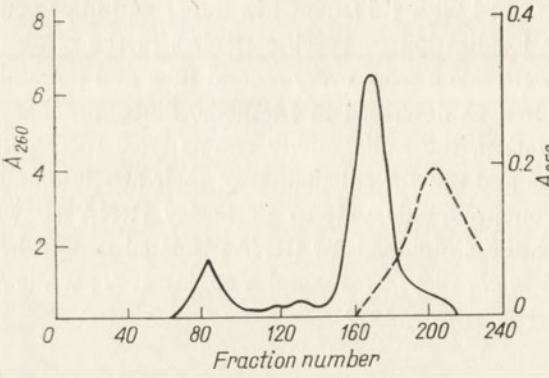


Fig. 3

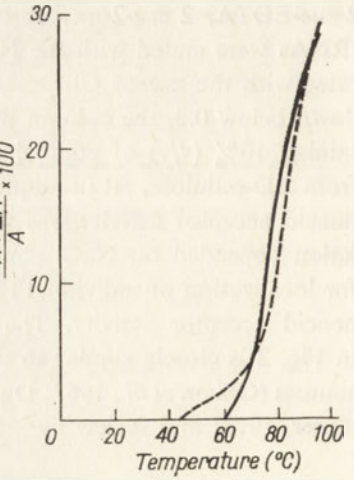


Fig. 4

Fig. 3. Gel filtration of crude barley tRNA on Sephadex G-100. tRNA, 1250 A_{260} units, was applied to a column (4 × 145 cm) equilibrated and eluted with 0.5 M-NaCl in 0.05 M-sodium-acetate buffer, pH 4.7, containing 10 mM-$MgCl_2$ and 2 mM-EDTA. RNA in the effluent was monitored at 260 nm (——) and the pigment at 350 nm (— — —). V_e/V_0 of the main RNA peak (4S RNA) was 1.8.

Fig. 4. Melting profiles of tRNA from barley (— — —) and yeast (——) in 50 mM-NaCl containing 10 mM-$MgCl_2$. Under the conditions indicated, T_m of barley tRNA was 81°C, and of yeast tRNA, 80°C.

under the same conditions, 80°C. These data correspond to those expected for native tRNAs (Cole *et al.*, 1972; Wittig *et al.*, 1973; Reeves *et al.*, 1970; Vanderhoef & Key, 1970). Thus, as it may be seen from the results presented, the procedure described enables isolation of relatively large quantities of native, undegraded tRNA even from the material containing an exceptionally high amount of ribonucleases.

REFERENCES

Avital, S. & Elson, D. (1969) *Biochim. Biophys. Acta*, **179**, 297 - 307.

Bellamy, A.R. & Ralph, R.K. (1968) in *Methods in Enzymology* (L. Grossman & K. Moldave, eds.) vol. 12, pp. 156 - 160. Academic Press, New York.

Cole, P.E., Jang, S.K. & Crothers, D.M. (1972) *Biochemistry*, **11**, 4358 - 4368.

Dudock, B.S., Katz, G., Taylor, E.K. & Holley, R.W. (1969) *Proc. Natl. Acad. Sci. U.S.A.*, **62**, 941 - 945.

Félix, F. & Chambon, H. (1974) *IX FEBS Meeting, Budapest, Abstr. Commun.* f10b37.

Gillam, I., Millward, S., Blow, D., von Tigerstrom, M., Wimmer, E. & Tener G.M. (1967) *Biochemistry*, **6**, 3043 - 3056.

Guderian, R.H., Pulliam, R.L. & Gordon, M.P. (1972) *Biochim. Biophys. Acta*, **262**, 50 - 65.

Kirby, K.S. (1956) *Biochem. J.*, **64**, 405 - 408.

Kothari, R.M. & Taylor, M.W. (1972) *J. Chromatogr.*, **73**, 479 - 501.

Labuda, D., Janowicz, Z., Haertlé, T. & Augustyniak, J. (1974) *Nucl. Acid Res.*, **1**, 1703 - 1712.

Legocki, A., Szymkowiak, A., Pech, K. & Pawełkiewicz, J. (1967) *Acta Biochim. Polon.*, **14**, 323 - 332.

Littauer, U.Z. & Inouye, H. (1973) *Ann. Rev. Biochem.*, **42**, 439 - 470.

Loening, U.E. (1967) *Biochem. J.*, **102**, 251 - 257.
Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.
Mans, R.J. & Novelli, G.D. (1961) *Arch. Biochem. Biophys.*, **94**, 48 - 53.
McDonald, M.R. (1955) in *Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, eds.) vol. 2, pp. 427 - 437. Academic Press, New York.
Pearson, R.L., Hancher, C.W., Weiss, J.F., Holladay, D.W. & Kelmers, A.D. (1973) *Biochim. Biophys. Acta*, **294**, 236 - 249.
Reeves, R.H., Cantor, C.R. & Chambers, R.W. (1970) *Biochemistry*, **9**, 3993 - 4001.
Robins, H.I. & McNutt, W.S. (1974) *Anal. Biochem.*, **57**, 636 - 639.
Robison, B. & Zimmerman, T.P. (1970) *Anal. Biochem.*, **37**, 11 - 20.
Shugart, L. (1972) *Exp. Gerontol.*, **7**, 251 - 262.
Stansly, P.G. & Seese, P.G. (1965) *Biochim. Biophys. Acta*, **95**, 671 - 674.
Vanderhoef, L.N. & Key, J.L. (1970) *Plant Physiol.*, **46**, 294 - 298.
White, B.N. & Tener, G.M. (1973) *Can. J. Biochem.*, **51**, 896 - 902.
Wittig, B., Reuter, S. & Gottschling, H. (1973) *Biochim. Biophys. Acta*, **331**, 221 - 230.
Yoshikami, D. & Keller, E.B. (1971) *Biochemistry*, **10**, 2969 - 2976.
Zubay, G. (1962) *J. Mol. Biol.*, **4**, 347 - 356.

IZOLACJA NA DUŻĄ SKALĘ tRNA Z ZARODKÓW JĘCZMIENIA

Streszczenie

1. Metoda izolacji na dużą skalę tRNA z zarodków jęczmienia obejmuje: ekstrakcję fenolem, odbiałczenie za pomocą mieszaniny chloroform - alkohol izoamylowy, sorpcję RNA na DEAE-celulozie, chromatografię na DEAE-celulozie i elucję tRNA w gradiencie stężenia NaCl oraz deaminoacylację tRNA w obecności bentonitu. Otrzymany tRNA nie zawiera domieszek białka i nie wykazuje aktywności RNazowej.

2. Aktywność akceptorowa, profil denaturacji termicznej, charakterystyka chromatograficzna na Sephadex oraz BD-celulozie wykazały podobieństwo tRNA z zarodków jęczmienia do tRNA otrzymanego z innych źródeł.

Received 27 June, 1975.

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 1

MARIA GAŁKA and W. OSTROWSKI

ALKALINE PHOSPHATASE OF *THIOBACILLUS THIOPARUS*. PARTIAL PURIFICATION AND PROPERTIES OF THE ENZYME

Institute of Medical Biochemistry, Nicolaus Copernicus Medical Academy, ul. Kopernika 7; 31-034 Kraków, Poland

Soluble alkaline phosphatase from *Thiobacillus thioparus* cells was purified about 230-fold. The enzyme had a mol. wt. of 50 000 daltons, optimum pH at 10.5, and was heat-resistant in the presence of diethanolamine.

Polyacrylamide-gel electrophoresis demonstrated contamination of the preparation with inactive proteins and the presence of two active bands.

The enzyme activity was distinctly stimulated by increasing concentrations of Tris or diethanolamine. In the presence of glycine, 1 mM-Zn^{2+} enhanced the enzyme activity; in Tris or diethanolamine buffers the activity was stimulated by 1 mM-Mg^{2+}, whereas Zn^{2+} had a strong inhibitory effect. Glycine at concentrations exceeding 25 mM also inhibited the enzyme.

Specificity of the enzyme is fairly broad.

Acid and alkaline phosphatases (EC 3.1.3.2. and 3.1.3.1, respectively) are widely distributed in bacteria, plant and animal tissues. In bacteria and yeast, location and activity of these enzymes display considerable variation, dependent on the species, age of culture and composition of growth media (Heppel, 1967; Thompson & MacLeod, 1974; Glenn & Mandelstam, 1971; Weimberg & Orton, 1965; Glew & Heath, 1971). Although phosphatases were investigated in various heterotrophic and autotrophic microorganisms, no information can be found in the literature concerning the chemosynthetic sulphur autotrophe, Thiobacillus.

In our earlier studies on nucleolytic enzymes in the cells of *Th. thioparus* (cf. Ostrowski *et al.*, 1970) phosphatase activity was also found in these bacteria, both at acid and alkaline pH values (Ostrowski & Walczak, 1961a,b). Preliminary investigations demonstrated that, while the acid phosphatase can be readily extracted from the disintegrated cells with 0.2 M-Tris buffer of pH 7.0, only a part of the alkaline phosphatase activity is transferred under these conditions into solution,

most of the activity remaining bound to the insoluble fragments of the cell membranes.

In this paper, partial purification and enzymic properties of the soluble alkaline phosphatase extracted from disrupted cells of *Th. thioparus*, are described.

MATERIALS AND METHODS

Reagents. Phenyl phosphate, disodium salt, creatine phosphate, sodium salt, bis-*p*-nitrophenyl phosphate, calcium salt, were from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); *p*-nitrophenyl phosphate and ascorbic acid were products of Merck (Darmstadt, G.F.R.), TEMED, bis-acrylamide, *o*-carboxyphenyl phosphate and 3′-CMP were from Koch-Light Lab. (Colnbrook, Bucks., England); ethanolamine phosphate was from Calbiochem (Los Angeles, Calif., U.S.A.); phenolphthaleine phosphate, sodium salt, was from P.Lewis Labs. Inc. (Milwaukee, Wis., U.S.A.); diethanolamine and sodium pyrophosphate were from POCh (Gliwice, Poland); Sephadex G-100, G-200 and DEAE-Sephadex A-50 were from Pharmacia (Uppsala, Sweden); cytochrome *c*, γ-globulin (human) and ceruloplasmin were from Wytwórnia Surowic i Szczepionek (Kraków, Poland); β-glycerophosphate, glutathione, reduced, and Tris were from B.D.H., Ltd. (Poole, Dorset, England); riboflavin flavoprotein was prepared from chicken egg-yolk after Ostrowski *et al.* (1968).

Bacterial cells. The strain of *Thiobacillus thioparus* was isolated and grown on inorganic medium as described by Ostrowski & Krawczyk (1957). The cells in the logarithmic phase of growth (96 h after inoculation) were harvested by filtration on Berkefeld filter, separated from the elemental sulphur by centrifugation and stored in the deep freeze.

Enzyme assays. The standard reaction mixture for the assay of alkaline phosphatase contained 10 mM-*p*-nitrophenyl phosphate as the substrate in 200 mM-Tris buffer, pH 10.0. The enzyme solution (100 µl) and substrate (100 µl) were incubated at 37°C for 60 min, then the reaction was terminated by adding 2.8 ml of 0.1 M-NaOH and the liberated *p*-nitrophenol was estimated at 400 nm. A unit of phosphatase activity is defined as the release of 1 nmole of *p*-nitrophenol/min. When other substrates were used, the activity was estimated by formation of inorganic phosphate (Lowry & Lopez, 1946).

Phosphodiesterase activity was assayed with 10 mM-bis-*p*-nitrophenyl phosphate in 200 mM-Tris-maleinate buffer, pH 8.8, containing 2 mM-$MnSO_4$ (Sinsheimer & Koerner, 1952). Other conditions were as in the assay of alkaline phosphatase.

5′-Nucleotidase was determined according to Neu (1967).

Electrophoresis. Disc electrophoresis was conducted in 7.5% polyacrylamide gel in Tris-glycine buffer, pH 8.3, ionic strength 0.45 for 2 h at 4°C at 3 mA per gel (Davis, 1964), samples of 100 µg protein being applied. Protein in the gel was stained with Amido Black and destained with 7% acetic acid containing 1% of Tween 80. The enzymic activity in the gel was located using *p*-nitrophenyl phosphate in 100 mM-Tris buffer, pH 10.0 (Dziembor *et al.*, 1971). Densitograms were obtained with an ERI 65 apparatus (Carl Zeiss, Jena, G.D.R.).

Starch block (40 × 8 × 1 cm) electrophoresis was carried out according to Lundquist *et al.* (1955) in 50 mM-Tris buffer, pH 8.2, containing 30 mM-NaCl. When the electrophoresis was completed (25 h at 5 V/cm, 4°C), the block was cut into 0.5 cm slices, eluted with 2 ml of water, and protein and enzymic activity determined.

Determination of molecular weight. The molecular weight of alkaline phosphatase was determined by filtration on Sephadex G-100 column (1.5 × 100 cm) according to Andrews (1964). The column was equilibrated with 20 mM-Tris buffer, pH 7.0, containing 100 mM-KCl, and the proteins were eluted with the same buffer solution at a rate of 10 ml/h.

Protein was determined according to Lowry *et al.* (1951).

RESULTS

Cell growth and enzyme activity. The activity of alkaline phosphatase in the suspension of washed cells, isolated at successive phases of growth of *Th. thioparus*, is presented in Fig. 1. The increase in enzyme activity parallelled the growth of the microorganism, and the activity was the highest after 96 hours of growth.

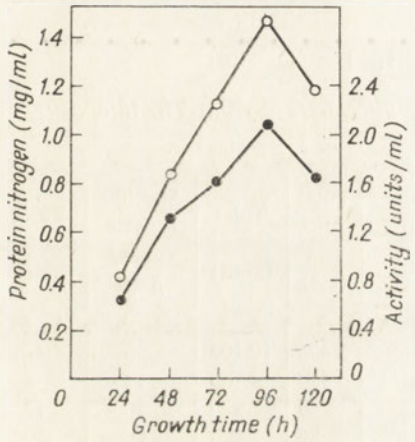


Fig. 1

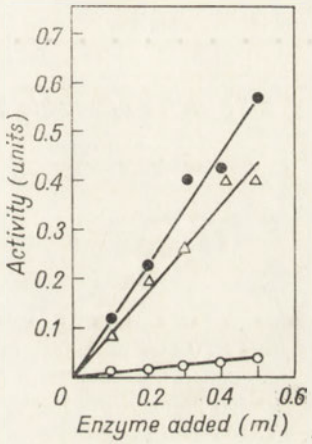


Fig. 2

Fig. 1. Activity of alkaline phosphatase in relation to cell growth in standard medium. At different time of growth the bacterial cells were isolated, washed, and the enzyme activity determined. For details see Methods. ○, Protein nitrogen per 1 ml of the medium; ●, enzyme activity.

Fig. 2. Activity of free and cell-bound alkaline phosphatase. Washed cells (1 mg protein N/ml) were disintegrated in 0.2 M-Tris buffer, pH 7.0, and the activity was measured in: ○, 20 000 *g* supernatant; △, insoluble material suspended in appropriate volume of buffer; ●, activity of the intact cell suspension.

Washed cells from the logarithmic phase were ground with carborundum powder in 0.2 M-Tris buffer, pH 7.0. After centrifugation at 20 000 *g* the activity of alkaline phosphatase was assayed in the supernatant and the insoluble fraction, and compared with the activity of intact cells. The results of the experiment presented in

Fig. 2 show the surface localization of the enzyme. Less than 10% of the activity was recovered in the supernatant of the disintegrated cells. It should be noted that the activity of intact *Th. thioparus* cells for hydrolysis of *p*-nitrophenyl phosphate showed two pH optima, at 5.5 and about 10.0.

Purification of soluble alkaline phosphatase from Th. thioparus. The purification procedure is outlined in Table 1. Bacteria harvested from about 1000 litres of bacterial culture and separated from elemental sulphur were ground with carborundum powder in a porcelain mortar in 0.2 M-Tris buffer, pH 7.0, in a cold-room. The suspension was centrifuged at 8000 *g*, the supernatant (about 800 ml) was fractionated with solid ammonium sulphate and the fraction precipitating at 0.3 - 0.55 saturation was collected by centrifugation, dissolved in a few millilitres of water and dialysed against water until a negative reaction for SO_4^{2-} ions was obtained. The enzyme solution (6 ml containing 60 mg of protein/ml) was applied in 2 ml portions to a Sephadex G-100 column (Fig. 3). The activity of alkaline phosphatase separated into three fractions, fraction II showing the highest specific activity. Fraction I eluted at V_0 of the column consisted of strongly aggregated proteins which were insoluble during extraction with salt solutions of different concentration and pH. Fraction III contained highly unstable enzyme which became completely inactivated during further purification steps.

Table 1

Purification of soluble alkaline phosphatase from Th. thioparus

The results are the averages of 3 experiments.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor	Yield (%)
Crude extract in 0.2 M-Tris buffer, pH 7.0	3 800	10 100	3	—	100
Ammonium sulphate fractionation (0.3 - 0.55 sat.)	350	4 700	13	4	47
Sephadex G-100 filtration	80	2 900	36	12	29
Sephadex G-200 filtration	26	2 500	96	30	25
1st Chromatography on DEAE-Sephadex A-50	6	1 880	303	95	19
2nd Chromatography on DEAE-Sephadex A-50	2	1 380	600	187	14
Starch block electrophoresis	1	743	705	231	7

The relatively stable fraction II was subjected to further purification. The eluates between 380 and 500 ml from three successive separations on Sephadex G-100 were pooled giving a total amount of 80 mg of the protein with the specific activity of 36 units/mg of protein. The solution was dialysed against water, concentrated by lyophilization to about 10 ml and then dialysed against 20 mM-Tris buffer, pH 7.0, containing 0.1 M-KCl. A sample (4 ml) containing 40 mg of the protein was adsorbed on a Sephadex G-200 column and eluted with the same buffer solution (Fig. 4). More than 90% of the applied enzyme activity emerged as a symmetrical peak between 80 and 120 ml of the effluent volume.

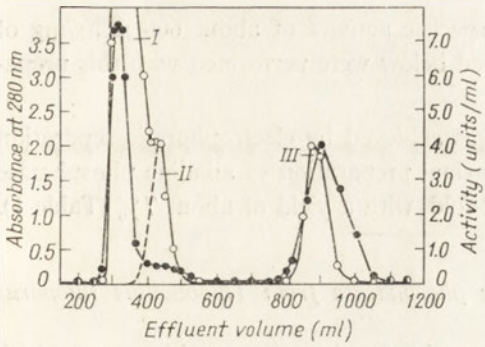


Fig. 3

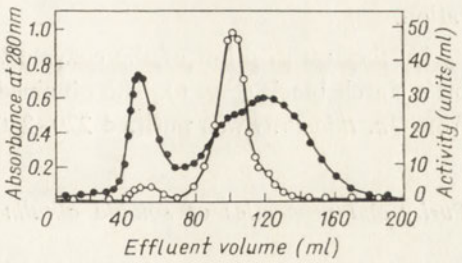


Fig. 4

Fig. 3. Sephadex G-100 column filtration of the fraction precipitated at 0.3 - 0.55 ammonium sulphate saturation. The column (2.3 × 135 cm) was equilibrated and eluted with 20 mM-Tris buffer, pH 7.0, the flow rate being 30 ml/h, and temperature 4°C. ○, Alkaline phosphatase activity; ●, $A_{280\ nm}$.

Fig. 4. Sephadex G-200 column filtration of fraction II from Sephadex G-100. The column (2 × × 54 cm) was equilibrated and eluted with 20 mM-Tris buffer, pH 7.0, the flow rate being 10 ml/h. ○, Alkaline phosphatase activity; ●, $A_{280\ nm}$.

Active fractions from two successive separations on Sephadex G-200 (26 mg of protein with the specific activity of 96 units) were dialysed against 20 mM-Tris buffer, pH 7.0, and adsorbed on a column (1.3 × 14 cm) loaded with DEAE-Sephadex A-50 and equilibrated with the above buffer solution. After washing the column with 300 ml of the same buffer, the elution was performed with a stepwise NaCl concentration gradient in the same buffer solution; the enzyme activity was eluted at 0.3 M-NaCl concentration.

The pooled active fractions were dialysed against 20 mM-Tris buffer, pH 7.0, adsorbed again on DEAE-Sephadex A-50 column, and eluted with a linear NaCl concentration gradient. The enzyme emerged between 0.2 and 0.3 M-NaCl (Fig. 5).

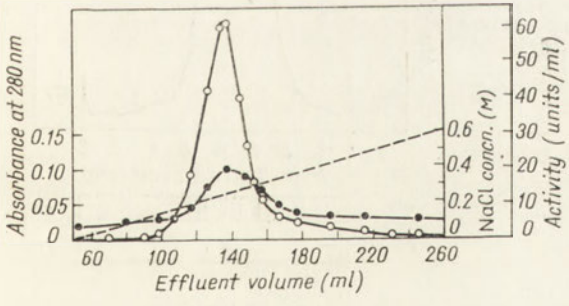


Fig. 5

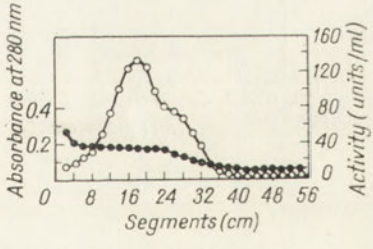


Fig. 6

Fig. 5. DEAE-Sephadex A-50 column chromatography of the active fractions from Sephadex G-200. The column (1.3 × 14 cm) was equilibrated with 20 mM-Tris buffer, pH 7.0, washed with 300 ml of the same buffer and then eluted with a linear NaCl concentration gradient. ○, Alkaline phosphatase activity; ●, $A_{280\ nm}$; — — —, NaCl gradient.

Fig. 6. Starch block electrophoresis of pooled active fractions from DEAE-Sephadex A-50. For details see Methods. ○, Alkaline phosphatase activity; ●, $A_{280\ nm}$.

The combined active fractions had the specific activity of about 600 units/mg of protein. Most of the experiments described below were performed with this preparation.

Further purification of the enzyme was achieved by electrophoretic separation on a starch block (Fig. 6). The obtained final preparation of alkaline phosphatase from *Th. thioparus* was purified 220-230-fold, with a yield of about 7% (Table 1).

Purity and properties of soluble alkaline phosphatase from Thiobacillus thioparus

Gel filtration and molecular weight. The active fractions separated by starch-block electrophoresis, dialysed against water and concentrated by lyophilization, gave on the column of Sephadex G-100 a symmetrical peak of the enzyme activity not coinciding with two major protein peaks. The molecular weight of the active peak, calculated from filtration on the calibrated Sephadex G-100 column, was 50 000 (Fig. 7).

Disc electrophoresis. Electrophoresis on polyacrylamide gel of the alkaline phosphatase preparation obtained by starch-block electrophoresis, with the specific activity exceeding 700 units/mg protein, showed a high content of inactive proteins (Fig. 8). The enzyme separated into two active fractions migrating toward anode at pH 8.3.

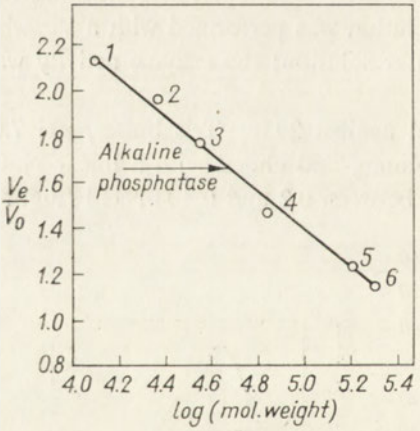


Fig. 7

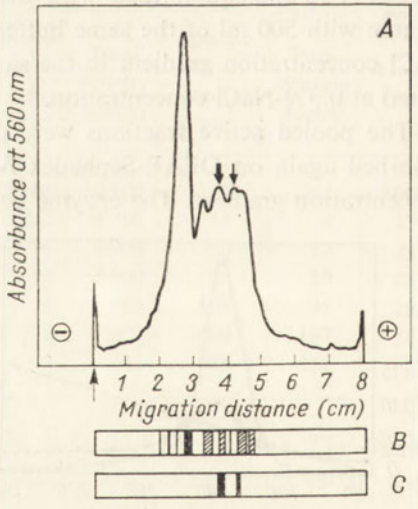


Fig. 8

Fig. 7. Molecular weight determination of alkaline phosphatase by Sephadex G-100 filtration. For details see Methods. Marker proteins: *1*, cytochrome *c*; *2*, trypsin; *3*, egg-yolk flavoprotein; *4*, bovine serum albumin; *5*, ceruloplasmin; *6*, γ-globulin (IgG).

Fig. 8. Polyacrylamide gel electrophoresis of the alkaline phosphatase preparation (705 units/mg protein). For details see Methods. *A*, Microdensitogram; *B*, protein bands; *C*, alkaline phosphatase zones.

In further experiments, the preparation obtained after second chromatography on DEAE-Sephadex A-50 (step 6) was used.

Optimum pH. The pH optimum for the activity of alkaline phosphatase was in glycine buffer 10.5 (Fig. 9), the activity in this buffer being markedly lower than in Tris or diethanolamine buffer.

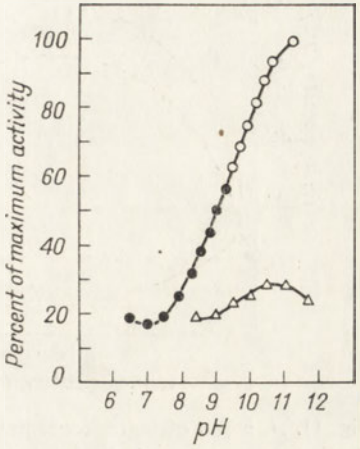


Fig. 9. Effect of pH on the activity of alkaline phosphatase. The activity was determined in 100 mM buffer solutions: ● Tris-HCl, pH 6.2 - 9.3; ○, Tris-NaOH, pH 9 - 11.2; and △, glycine-NaOH, pH 8.3 - 11.7, and with 2 mM-*p*-nitrophenyl phosphate as substrate.

Enzyme activity in different buffer solutions. At pH 10.0, in all three buffers used the initial rate of *p*-nitrophenol liberation was linear with time for at least 2 h, and with enzyme concentration at least up to 40 µg/sample (Fig. 10). The enzyme activity was determined at buffer concentration of 100 mM, and was the lowest in glycine buffer. The effect of buffer concentration is presented in Fig. 11A. The highest enzyme activity was found at about 500 mM-Tris and 800 mM-diethanolamine concentrations, while glycine at 25 mM concentration slightly activated the enzyme,

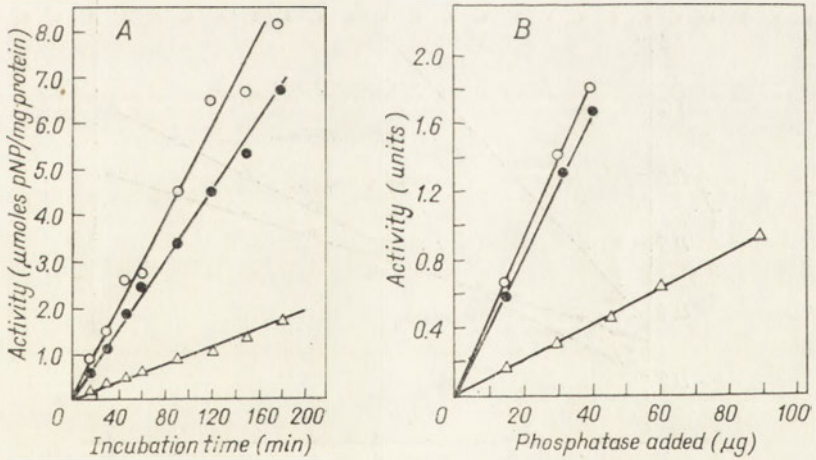


Fig. 10. The effect of incubation time (*A*) and enzyme concentration (*B*) on the rate of *p*-nitrophenyl phosphate hydrolysis in different 100 mM buffer solutions, pH 10.0. The reaction mixture contained 5 mM-*p*-nitrophenyl phosphate and in *A*, 60 µg of enzyme protein; in *B*, the reaction time was 60 min. Buffers used: ○, diethanolamine; ●, Tris; △, glycine.

and at higher concentrations strongly inhibited the activity. K_i for glycine in 500 mM-Tris buffer, pH 10.0, determined from the Lineweaver-Burk plot, was 0.68 mM, and the inhibition was non-competitive (Fig. 11B).

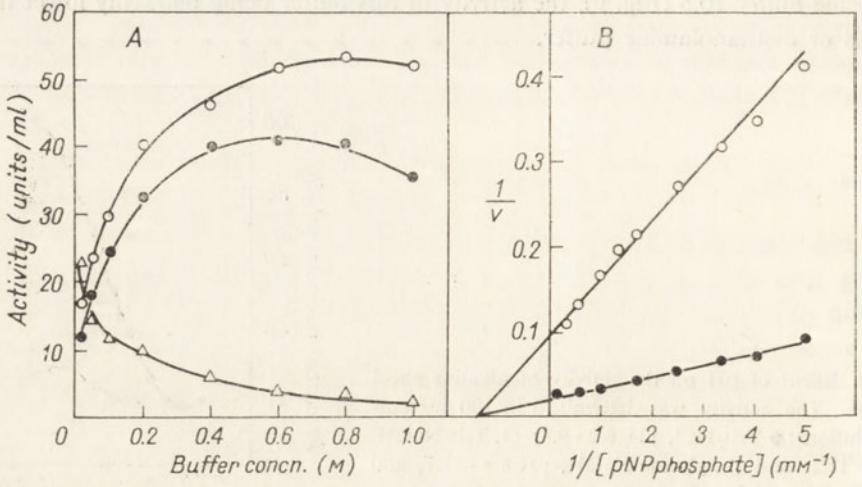


Fig. 11. *A*, Effect of buffer concentration on the activity of alkaline phosphatase at pH 10.0 in: ○, diethanolamine; ●, Tris; △, glycine. *B*, Lineweaver-Burk plot for the inhibition of alkaline phosphatase by glycine; the reaction was carried out in: ●, 500 mM-Tris buffer alone, and ○, supplemented with 500 mM-glycine

K_m values for *p*-nitrophenyl phosphate as the substrate at pH 10.0 in 100 mM-glycine, Tris and diethanolamine buffer solutions were 0.62, 0.19 and 0.48 mM, respectively (Fig. 12).

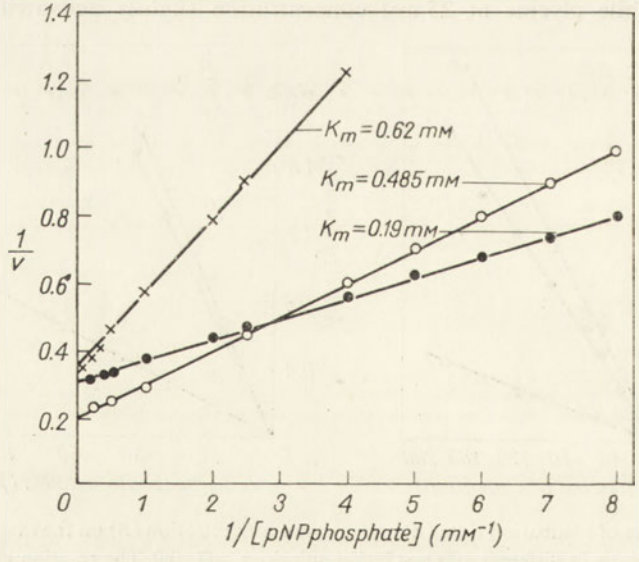


Fig. 12. Effect of substrate concentration on the rate of *p*-nitrophenyl phosphate hydrolysis in 100 mM buffer solutions at pH 10.0: ○, diethanolamine; ●, Tris; ×, glycine.

Substrate specificity. Relative activities of the enzyme toward several phosphate esters, are presented in Table 2. It is interesting that the enzyme showed the highest activity toward phenyl phosphate and 5′-AMP. Hydrolysis was the lowest for creatine phosphate and phenolphthaleine phosphate. The enzyme hydrolysed also purine and pyrimidine di- and triphosphonucleosides but at a lower rate than the corresponding monophosphonucleosides. Among phosphate esters of carbohydrates, the highest hydrolysis was observed for fructose-1,6-diphosphate.

Table 2

Substrate specificity of alkaline phosphatase from Th. thioparus

The reaction mixture contained 5-mM substrate and 100 mM-Tris buffer, pH 10.0. Enzyme activity was determined by estimation of inorganic phosphate.

Substrate	Relative activity (%)	Substrate	Relative activity (%)
p-Nitrophenyl phosphate	100	3′-AMP	52
Phenyl phosphate	141	5′-AMP	111
β-Glycerophosphate	37	ADP	77
α-Naphthyl phosphate	79	ATP	43
β-Naphthyl phosphate	59	5′-GMP	80
Phosphoethanolamine	29	GDP	54
Creatine phosphate	8	3′-UMP*	59
Phenolphthaleine phosphate	22	3′-CMP*	67
o-Carboxyphenyl phosphate	26	5′-CMP	64
Inorganic pyrophosphate	31	CDP	59
bis-*p*-Nitrophenyl phosphate	39	Glucose-1-phosphate	52
		Glucose-6-phosphate	32
p-Nitrophenyl thymidine-3′-phosphate	4	Fructose-6-phosphate	41
		Fructose-1,6-diphosphate	80

* Mixtures of 2′-and 3′-phosphates.

The alkaline phosphatase hydrolysed also phosphodiester compounds such as bis-*p*-nitrophenyl phosphate, and to some extent *p*-nitrophenyl thymidine 3′-phosphate. The enzyme preparation was probably contaminated by pyrophosphatase and phosphodiesterase present in this microorganism (Ostrowski, 1961).

Effect of ions and other substances. The effect of various substances on the activity of alkaline phosphatase was investigated in different buffer solutions, with *p*-nitrophenyl phosphate as substrate (Table 3). Zn^{2+} and Cd^{2+} at 1 mM concentration inhibited the activity by more than 80% in Tris and diethanolamine buffers. On the other hand, in glycine buffer 1 mM-Zn^{2+} activated the enzyme about 2.5-fold, whereas at higher Zn^{2+} concentration the enzyme activity decreased (Fig. 13). At 1 mM concentration Mg^{2+} ions enhanced the enzyme activity in all three buffer solutions, and other divalent ions showed unspecific effect. EDTA (1 mM) inhibited almost completely the enzyme activity, which in glycine buffer could be restored by Zn^{2+} and in Tris or diethanolamine buffer by Mg^{2+} (Fig. 14).

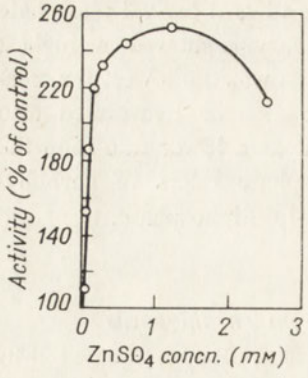


Fig. 13

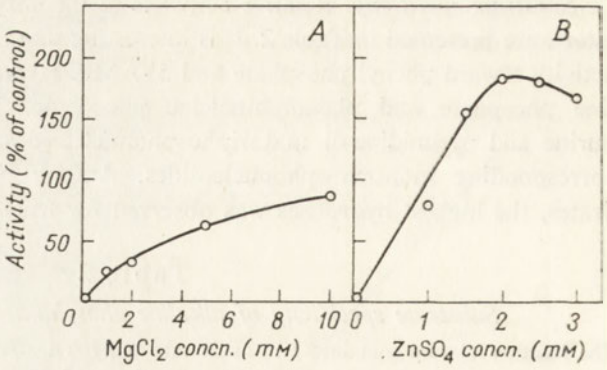


Fig. 14

Fig. 13. Effect of Zn^{2+} ion concentration on the activity of alkaline phosphatase in 100 mM-glycine buffer, pH 10.0. The enzyme preparation was first dialysed against deionized water, then the activity was determined with 2 mM-*p*-nitrophenyl phosphate as substrate.

Fig. 14. Reactivation by Mg^{2+} or Zn^{2+} of alkaline phosphatase inhibited by EDTA. The enzyme was assayed with 2 mM-*p*-nitrophenyl phosphate in the presence of 1 mM-EDTA at pH 10.0 in: *A*, 100 mM-Tris or *B*, 100 mM-glycine buffer. The activity without EDTA is taken as 100.

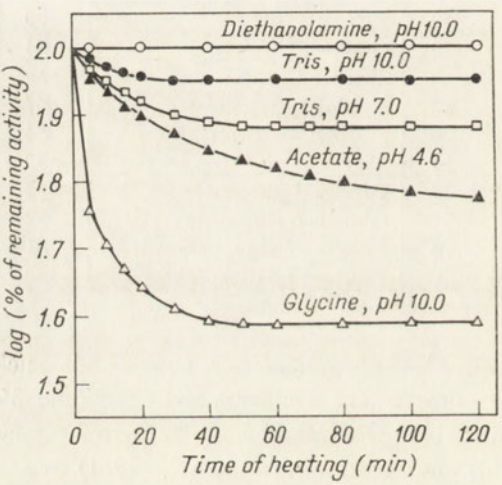


Fig. 15

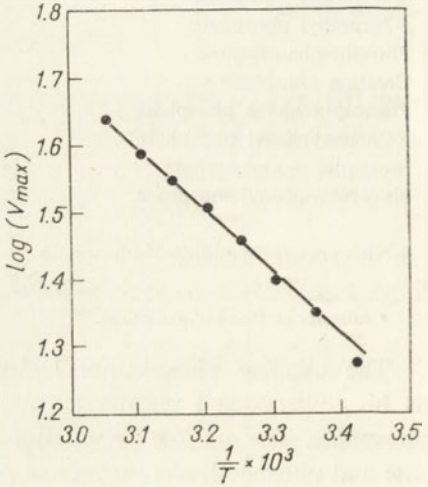


Fig. 16

Fig. 15. Rates of inactivation of alkaline phosphatase at 55°C in various buffer solutions at the indicated pH values. About 300 μg of enzyme in 1 ml was incubated with the indicated buffer solution, 100 mM, cooled to 0°C, then the activity was determined under standard conditions.

Fig. 16. Arrhenius plot for the effect of temperature on the activity of alkaline phosphatase in 100 mM Tris buffer, pH 10.0.

Cysteine, glutathione and 2-mercaptoethanol at 10 mM concentrations inhibited alkaline phosphatase, in most cases by more than 50%. Fluoride and tartrate had no effect (Table 3).

Table 3

Effect of various metal ions and other compounds on the activity of alkaline phosphatase from Th. thioparus in different buffer solutions

All compounds were first preincubated at 37°C for 15 min with 40 units of the enzyme in 100 mM appropriate buffer solution, pH 10.0 (100 µl), then equal volume of 10 mM-*p*-nitrophenyl phosphate was added and the mixture incubated for 60 min.

Compound added	Concentration (mM)	Relative activity (%)		
		Diethanolamine	Tris	Glycine
None (control)	0	100	100	100
$MgCl_2$	1	140	175	118
$MnSO_4$	1	68	75	128
$ZnSO_4$	1	19	18	235
$CoCl_2$	1	51	66	94
$CuSO_4$	1	67	80	97
$BaCl_2$	1	101	109	85
$CaCl_2$	1	93	109	93
$CdSO_4$	1	14	16	52
$Ni(NO_3)_2$	1	26	51	93
$FeSO_4$	1	109	100	100
$(Fe)_2(SO_4)_3$	1	114	75	100
NaF	1	111	107	—
NaF	10	108	100	—
Tartrate	10	91	104	—
EDTA	1	4	5	0
EDTA	10	2	3	0
Cysteine	10	15	28	50
Glutathione (reduced)	10	31	43	91
2-Mercaptoethanol	10	35	58	84
Iodoacetate	1	87	107	90

Heat inactivation, temperature optimum and energy of activation. The effect of heating at 55°C on the activity of alkaline phosphatase was studied in different buffers and at different pH values (Fig. 15). At pH 10.0 in diethanolamine buffer the enzyme did not lose its activity even after 2 hours of heating. However, at the same pH, the enzyme lost 60% of the initial activity after 30 min heating in glycine buffer. At pH 4.6 in acetate buffer and at pH 7.0 in Tris, after 30 min of heating the phosphatase retained 75 and 80%, respectively, of the initial activity.

The temperature optimum for the enzyme in 100 mM-Tris buffer, pH 10.0, was 58°C. The Arrhenius plot of the reaction rate gave a straight line over the temperature range studied (Fig. 16). The value for the energy of activation was 4500 cal/mole.

DISCUSSION

Thiobacillus thioparus is a small, gram-negative, chemoautotrophic eubacterium which oxidizes divalent sulphur atom of thiosulphate to elemental sulphur and polythionates (Baalsrud, 1954). Due to the theoretical and practical importance

of sulphur autotrophes, this microorganism was a subject of numerous metabolic studies (Roy & Trudinger, 1970). However, not much attention was paid to the presence and properties of phosphomonoesterases, which in the cell metabolism of sulphur bacteria perform probably regulative function in the processes of oxidation of inorganic sulphur compounds, in the transport of different metabolites and in the control of orthophosphate concentration in the cell (Marunouchi, 1969; Tominaga & Mori, 1974).

The results presented in this paper indicate that the alkaline phosphatase of *Th. thioparus* is located mainly outside the cytoplasmic cell membrane. In spite of the treatment of disintegrated cells with detergents, lysozyme, and the use of buffers of different pH and ionic strength, not more than 10% of the enzyme activity could be transferred into solution. In this respect the alkaline phosphatase of *Th. thioparus* is similar to the enzyme of other gram-negative bacteria (Glenn & Mandelstam, 1971; Thompson & MacLeod, 1974), as well as of some tumour cells (Wolpert *et al.*, 1971; Lee & Sartorelli, 1974).

Fractionation of the cell extract of *Th. thioparus* on Sephadex G-100 column gave three active peaks differing in specific activity and stability. The alkaline phosphatase II, which had the highest specific activity and was most heat-resistant, was further purified on Sephadex G-200, DEAE-Sephadex A-50 and by electrophoresis on starch block. The more than 220-fold purified enzyme preparation was still heterogeneous on polyacrylamide disc gel electrophoresis and separated into two active fractions with molecular weight of 50 000 and optimum activity at pH about 10.

The enzyme preparation purified partially (about 180-fold) hydrolysed also, at considerable rates, di- and tri-phosphonucleosides, which may indicate contamination by pyrophosphatase. However, the possibility that phosphomonoesterase and phosphodiesterase activities may be due to the same enzyme protein, should also be considered.

The activity of partially purified alkaline phosphatase was much lower in glycine buffer than in Tris or diethanolamine buffer, and glycine at concentrations exceeding 25 mM inhibited the enzyme activity. In Tris and diethanolamine buffers the activity increased with buffer concentration up to 800 mM and 500 mM, respectively. Since both these buffers are good acceptors of P_i, the observed enhanced activity could be due to transphosphorylation between the buffer and the *p*-nitrophenyl phosphate substrate (Dayan & Wilson, 1964; Wilson *et al.*, 1964; Glew & Heath, 1971; Yoshizumi & Coleman, 1974).

In glycine buffer, Zn^{2+} at 1 mM concentration was an activating factor, whereas in Tris and diethanolamine buffers, zinc ions at the same concentration had a strong inhibitory effect. This property of the *Th. thioparus* alkaline phosphatase is similar to that of some microbial (Glenn & Mandelstam, 1971; Glew & Heath, 1971; Csopak & Szajn, 1973) and mammalian alkaline phosphatases (Hofstee, 1955; Harkness, 1968; Fernley, 1971).

The complex mechanism of glycine action on the *Th. thioparus* phosphatase is not clear. Glycine at higher concentration may prevent the breakdown of the formed phosphoryl-enzyme intermediate or cause conformational changes of the enzyme

protein as a result of specific modification of its active site, as it was suggested for placental alkaline phosphatase and phenylalanine as an inhibitor (Fernley & Walker, 1970). The behaviour of the enzyme in glycine buffer may also suggest that the phosphoryl-enzyme complex has considerably higher affinity to water than to glycine during the dephosphorylation step, and that higher glycine concentrations may even inhibit the formation of E-P complex.

Chelating agents such as EDTA and cysteine are potent inhibitors of the *Th. thioparus* enzyme. EDTA caused a sharp decrease in activity, and the enzyme was completely inactivated when the concentration of the inhibitor approached 1 mM. Full activity of the EDTA-treated phosphatase was restored when an excess of Mg^{2+} (in Tris buffer) or Zn^{2+} (in glycine buffer) was added. These results suggest that Mg^{2+} (or Zn^{2+}) is a divalent cation essential for the activity of the alkaline phosphatase from *Th. thioparus*, which resembles in this respect the alkaline phosphatase of *E. coli* (Schlesinger & Barrett, 1965; Petitclerc *et al.*, 1970).

The *Th. thioparus* enzyme shows distinct specificity for the alcohol group of the phosphate monoester substrates, differing in its behaviour from other alkaline phosphatases.

REFERENCES

Andrews, P. (1964) *Biochem. J.* **91**, 222 - 233.

Baalsrud, K. (1954) *Autotrophic Microorganisms*, pp. 54 - 67. University Press, Cambridge.

Csopak, H. & Szajn, H. (1973) *Arch. Biochem. Biophys.*, **157**, 374 - 379.

Davis, B.J. (1964) *Ann. N. Y. Acad. Sci.*, **121**, 404 - 427.

Dayan, J. & Wilson, I.B. (1964) *Biochim. Biophys. Acta*, **81**, 620 - 623.

Dziembor, E., Gryszkiewicz, J. & Ostrowski, W. (1971) *Acta Biochim. Polon.*, **18**, 419 - 431.

Fernley, H.N. (1971) in *The Enzymes* (Boyer, P.D., ed.) vol. 4, pp. 417 - 447. Academic Press, New York.

Fernley, H.N. & Walker, P.G. (1970) *Biochem. J.*, **116**, 543 - 544.

Glenn, A.R. & Mandelstam, J. (1971) *Biochem. J.*, **123**, 129 - 138.

Glew, R.H. & Heath, E.C. (1971) *J. Biol. Chem.*, **246**, 1556 - 1565.

Harkness, D.R. (1968) *Arch. Biochem. Biophys.*, **126**, 503 - 512.

Heppel, L.A. (1967) *Science*, **156**, 1451 - 1455.

Hofstee, N.H.J. (1955) *Arch. Biochem. Biophys.*, **59**, 352 - 365.

Lee, M.H. & Sartorelli, A.C. (1974) *Biochim. Biophys. Acta*, **358**, 69 - 81.

Lowry, O.H. & Lopez, J.A. (1946) *J. Biol. Chem.*, **162**, 421 - 428.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.

Lundquist, F., Thosteinsson, Th. & Buus, O. (1955) *Biochem. J.*, **59**, 69 - 79.

Marunouchi, T. (1969) *J. Biochem. (Tokyo)*, **66**, 113 - 115.

Neu, H.C. (1967) *J. Biol. Chem.*, **242**, 3896 - 3904.

Ostrowski, W. (1961) *Experientia*, **17**, 398 - 400.

Ostrowski, W. & Krawczyk, A. (1957) *Acta Biochim. Polon.*, **4**, 249 - 265.

Ostrowski, W. & Walczak, Z. (1961a) *Acta Biochim. Polon.*, **8**, 345 - 361.

Ostrowski, W. & Walczak, Z. (1961b) *V Intern. Congress of Biochemistry, Moscow, Abstract No.* 5/- 115·1367.

Ostrowski, W., Żak, Z. & Krawczyk, A. (1968) *Acta Biochim. Polon.*, **15**, 241 - 260.

Ostrowski, W., Walczak, Z., Stasiuk, L. & Laskowski, M. (1970) *Acta Biochim. Polon.*, **17**, 73 - 87.

Petitclerc, C., Lazdunski, C., Chappelet, D., Moulin, A. & Lazdunski, M. (1970) *Eur. J. Biochem.*, **14**, 301 - 308.

Reid, T.W. & Wilson, I.B. (1971) in *The Enzymes* (Boyer, P.D., ed.) vol. 4, pp. 373 - 415. Academic Press, New York.
Roy, A.B. & Trudinger, P.A. (1970) *The Biochemistry of Inorganic Compounds of Sulphur*, pp. 207 - 250. University Press, Cambridge.
Schlesinger, M.J. & Barrett, K. (1965) *J. Biol. Chem.*, **240**, 4284 - 4292.
Sinsheimer, R.L. & Koerner, J.F. (1952) *J. Biol. Chem.*, **198**, 293 - 296.
Thompson, L.M.M. & MacLeod, R.A. (1974) *J. Bacteriol.*, **117**, 813 - 818.
Tominaga, N. & Mori, T. (1974) *J. Biochem. (Tokyo)*, **76**, 397 - 408.
Walczak, Z. & Ostrowski, W. (1964) *Acta Biochim. Polon.*, **11**, 241 - 252.
Weimberg, R. & Orton, W.L. (1965) *J. Bacteriol.*, **90**, 82 - 94.
Wilson, I.B., Dayan, J. & Cyr, K. (1964) *J. Biol. Chem.*, **239**, 4182 - 4185.
Wolpert, M.K., Damle, S.P., Brown, J.E., Sznycer, E., Agrawal, K.C. & Sartorelli, A.C. (1971) *Cancer Res.*, **31**, 1620 - 1626.
Yoshizumi, F.K. & Coleman, J.E. (1974) *Arch. Biochem. Biophys.*, **160**, 255 - 268.

FOSFATAZA ZASADOWA *THIOBACILLUS THIOPARUS*. CZĘŚCIOWE OCZYSZCZANIE I WŁASNOŚCI ENZYMU

Streszczenie

Rozpuszczalną fosfatazę zasadową wyekstrahowaną z komórek *Th. thioparus*, oczyszczono ok. 230-krotnie. Ciężar cząsteczkowy enzymu oznaczony metodą filtracji na kolumnie z Sephadexem G-100 wynosi 50 000, optimum pH 10.5; enzym jest odporny na ogrzewanie w obecności dwuetanoloaminy.

Elektroforeza preparatu na żelu poliakryloamidowym wykazuje dwa aktywne pasma enzymu wędrujące do anody przy pH 8,3 oraz szereg nieaktywnych białek.

Aktywność enzymu znacznie wzrasta w obecności Tris i dwuetanoloaminy, podczas gdy glicyna w stężeniu powyżej 25 mM hamuje badaną fosfatazę. W obecności glicyny, 1 mM-Zn^{2+} aktywuje enzym, natomiast w obecności Tris i dwuetanoloaminy cynk jest silnym inhibitorem. Jony Mg^{2+} (1 mM) aktywują enzym w buforach Tris i dwuetanoloaminowym.

Fosfataza alkaliczna *Th. thioparus* hydrolizuje liczne estry kwasu fosforowego.

Received 24 July, 1975.

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 1

M. KULISZEWSKI, MARIA SZUMIŁO, IWONA RAHDEN and W. SENDECKI

THE EFFECT OF UNILATERAL NEPHRECTOMY ON THE ACTIVITY OF RIBOSOMES IN THE REMAINING KIDNEY*

Department of Biochemistry, Institute of Biopharmacy, Medical School ul. Banacha 1; 02-097 Warszawa, Poland

1. Ribosomes isolated from hypertrophic kidneys were examined at different time intervals after unilateral nephrectomy. The activity of ribosomes measured by [^{14}C] leucine incorporation and synthesis of polyphenylalanine from [^{14}C]Phe-tRNA, was increased after 24 h, whereas changes in the composition of ribosomal protein fractions appeared as early as within 6 hours after the operation.

2. In hypertrophic kidney the content of ribosomes increased but the ratio of free and membrane-bound ribosomes remained practically unaltered. However, the specific activity of free ribosomes was twice as high as that of bound ribosomes.

3. It is concluded that the enhanced activity of free ribosomes is one of the factors responsible for the increased protein synthesis in hypertrophic kidney.

After unilateral nephrectomy, the remaining kidney undergoes hypertrophy and hyperplasia (see: Nowiński & Goss, 1969). The compensatory hypertrophy is accompanied by an increase in the content of cellular DNA, RNA and protein (see: Halliburton, 1969). The increase in the amount of RNA and protein is due both to their enhanced synthesis and decreased degradation (Malt & Lemaitre, 1968; Willems *et al.*, 1969; Hill & Malamud, 1974; Hill *et al.*, 1974; Hill, 1975).

It was demonstrated previously (Sendecki *et al.*, 1973a) that the ribosomes isolated from hypertrophic kidney at 48 hours after unilateral nephrectomy, exhibited increased activity in amino acids incorporation. At the same time, the aminoacyl-tRNA binding factor was also more active (Sendecki *et al.*, 1973b).

The aim of the present work was to investigate the mechanism of the increased activity of ribosomes from the kidney undergoing compensatory hypertrophy.

* This work was supported by the Polish Academy of Sciences within the project 09.3.1.

MATERIALS AND METHODS

Special reagents. L-[U-^{14}C]Phenylalanine (spec. act. 0.1 mCi/0.088 mg) and L-[U-^{14}C]leucine (spec. act. 0.1 mCi/0.105 mg) were from UVVVR (Praha, Czechoslovakia). Polyuridylic acid was from Miles Lab. Inc. (Elkhart, Ind., U.S.A.). GTP, ATP, dithiothreitol (DTT) and 19 non-radioactive amino acids were from Calbiochem (Lucerne, Switzerland).

Tris, PPO, POPOP, acrylamide and *N,N'*-methylenediacrylamide were products of Koch-Light Lab. Ltd (Colnbrook, Bucks., England); *N,N,N',N'*-tetramethylethylenediamine was from Fluka AG (Buchs SG, Switzerland), *β*-alanine from B.D.H. Chemicals Ltd (Poole, Dorset, England) and Amido Black 10B from Reachim (Moscow, U.S.S.R.).

Phosphoenolpyruvic acid and pyruvate kinase (EC 2.7.1.40) were purchased from Boehringer und Soehne (Mannheim, G.F.R.).

Bentonite was from Fischer Scientific Comp. (Fair Lawn, N.J., U.S.A.). Sephadex G-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Millipore filters H.A. (0.45 μ) were from Millipore Corp. (Bedford, Mass., U.S.A.), and glass-fibre paper Whatman GF-83 from W.R. Balston (Springfield Mill, Maidstone, Kent, England).

All other reagents were of Polish origin.

Animals. Male albino rats weighing 130-180 g were used for the experiments. The animals were fed with standard LSM mixture (Bacutil, Warszawa) and given water *ad libitum*. Unilateral nephrectomy was performed as described by Sendecki *et al.* (1972). At determined time intervals after the operation the animals were killed by cervical dislocation and the remaining hypertrophic kidney was isolated and placed in ice-cold buffer. All further steps were carried out at 2 - 4°C.

Isolation of total ribosomes. The cooled kidney was homogenized in a Potter-Elvehjem Teflon-glass homogenizer (5 × 10 sec at 1000 rev./min) in 2 vol. of buffer *A* composed of: 50 mM-Tris-HCl, pH 7.6, 80 mM-KCl, 4 mM-$MgCl_2$, 6 mM-2-mercaptoethanol and 0.25 M-sucrose. The buffer mixture contained bentonite (16 mg/g fresh tissue weight) to inhibit the RNase activity (Sendecki *et al.*, 1972). Nuclei and mitochondria were sedimented by centrifugation for 15 min at 15 000 *g* in a Sorvall centrifuge (ss 34 rotor). Then the supernatant was centrifuged in an angle rotor of MSE Superspeed 65 ultracentrifuge for 90 min at 150 000 *g*. The ribosome pellet was suspended in buffer *A*, to which a freshly prepared solution of sodium deoxycholate was added to a concentration of 1%, and the mixture centrifuged again for 2 h at 150 000 *g*. The obtained ribosomes were washed with buffer *A* in which the KCl concentration was brought to 0.5 M, in order to remove cytoplasmic proteins (Moldave & Skogerson, 1967). The KCl-washed ribosomes were sedimented by centrifugation through 1 M-sucrose in buffer *A* for 2 h at 200 000 *g*. The sedimented ribosomes were suspended in buffer *A* containing 25% glycerol, and stored in small portions at —20°C.

Only those ribosomal preparations in which the ratio E_{260}/E_{235} exceeded 1.7, and E_{260}/E_{280} was higher than 1.4, were used for the experiments. One $E^{1\,cm}_{260\,nm}$

unit was accepted as corresponding to 80 μg of ribosomes/ml (Trachewsky *et al.*, 1972).

Isolation of free and membrane-bound ribosomes was performed according to Glazer & Sartorelli (1972). The kidney was homogenized in buffer *B* (50 mM-Tris-HCl, pH 7.8, 50 mM-KCl, 5 mM-$MgCl_2$) containing bentonite (16 mg/g fresh tissue weight). The homogenization and centrifugation were carried out as described above. The post-mitochondrial supernatant was applied to a discontinuous sucrose density gradient (0.5 and 2.0 M) and centrifuged in an angle rotor of MSE Superspeed 65 ultracentrifuge for 4 h at 200 000 *g*. The sediment contained free ribosomes; the bound ribosomes were isolated from the interphase. The obtained preparations were stored in buffer *B* containing 4 mM-dithiothreitol and 25% glycerol.

Isolation of partially purified cytoplasmic factors EF-1 and EF-2 from liver of intact animals. The cytoplasmic factors were isolated and purified according to Moldave *et al.* (1971) from the "pH 5 supernatant" of the post-ribosomal fraction.

Preparation of [^{14}C]*Phe-tRNA.* The "pH 5 sediment" (see above) containing tRNA's and aminoacyl-tRNA synthetases, was used for preparation of [^{14}C]Phe-tRNA (Moldave, 1963).

Ribosomal proteins were extracted with 4 M-urea containing 2 M-lithium chloride as described by Spitnik-Elson (1965).

Polyacrylamide-gel electrophoresis was performed at pH 4.5 according to Gesteland & Staehelin (1967). The tubes (140 × 6 mm) were filled with 10% gel to a height of 110 mm and the electrophoresis was run for 5 h at 2.5 mA/tube at 4°C. The electrophoretograms were stained with 0.5% Amido Black in 7.5% acetic acid for 1 h, destained by extensive washing with 7.5% acetic acid, and scanned with a densitometer ERI-65 (Carl Zeiss, Jena), adapted for analysis of polyacrylamide-gel slabs.

Ribonucleic acid was determined according to Mejbaum (Campbell & Sargent, 1969) with yeast RNA (Research Centre of the Medical School, Łódź, Poland) as standard.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin, Fraction V (Pentex, Kankakee, Ill., U.S.A.) as standard.

Incorporation of [^{14}C]*leucine* in the cell-free system was carried out as outlined in the legend to Fig. 2.

Synthesis of [^{14}C]*polyphenylalanine from* [^{14}C]*Phe-tRNA.* This was carried out as described by Sendecki *et al.* (1973a) and outlined in the legend to Table 1.

Radioactivity measurements were carried out as described by Sendecki *et al.* (1973a).

RESULTS

Activity of the total ribosome fraction

The changes in the activity of ribosomes from hypertrophic kidney in relation to the time elapsed after unilateral nephrectomy, were investigated in the amino acid incorporating cell-free system containing cytoplasmic factors from liver of control animals. It was found that after 6 and 12 hours the synthesis of polyphe-

nylalanine from [^{14}C]Phe-tRNA proceeded at a rate similar to that observed with ribosomes from the kidney of intact or sham-operated animals. After 24 hours an increase in the activity was clearly visible, and after 48 hours the activity was twice as high as in the control (Table 1).

Table 1

Synthesis of [^{14}C]*polyphenylalanine from* [^{14}C]*Phe-tRNA by ribosomes from control and hypertrophic kidney*

The incubation mixture contained in a volume of 0.25 ml: 50 mM-Tris-HCl, pH 7.6; 6 mM-$MgCl_2$; 80 mM-KCl; 0.2 mM-GTP; 4 mM-dithiothreitol; 100 μg of poly(U); [^{14}C]Phe-tRNA (8000 d.p.m.); cytoplasmic factors from liver (400 μg protein) and 100 μg of ribosomes from control or hypertrophic kidney. The incubation was carried out for 20 min at 37°C, and stopped by adding 3 ml of 10% trichloroacetic acid. The sample was left for 3 - 4 h at 4°C, then heated for 7 min at 90°C in a water bath. The precipitate was collected on Millipore filter, washed 3 times with 5% trichloroacetic acid and the radioactivity was determined.

Time after nephrectomy (h)	Incorporation of [^{14}C]Phe (pmoles/mg of ribosomes)	Increasing factor
Control	8.0	—
6	7.0	0.9
12	8.0	1.0
24	12.0	1.5
48	17.0	2.1

Increased activity of ribosomes in regenerating liver has been reported to be related to changes in ribosomal proteins (Liew & Gornall, 1973; Gressner & Wool, 1974; Scheinbuks *et al.*, 1974; Anderson *et al.*, 1975). To see whether the same holds for the kidney undergoing compensatory hypertrophy, the ribosomal proteins were isolated and submitted to one-dimensional polyacrylamide-gel electrophoresis (Fig. 1). The proteins separated into at least 20 bands, the electrophoretic pattern of the proteins extracted from ribosomes of hypertrophic kidney being more complex than that for the control. An additional protein band showing high electrophoretic mobility appeared beginning with 6 h after the operation. After 48 h, quantitative changes in the particular fractions were also observed.

Free and membrane-bound ribosomes

Free ribosomes are generally considered to be involved in the synthesis of intracellular proteins, whereas proteins designated for export are synthesized on bound ribosomes (see Campbell & Lawford, 1967). Since during compensatory kidney hypertrophy the requirement for intracellular protein is increased, a change in the proportion of free and bound ribosomes could be expected.

The analysis of the distribution of ribosomes into these two forms showed, in agreement with Blobel & Potter (1967) that in kidney the ribosomes occurred mostly

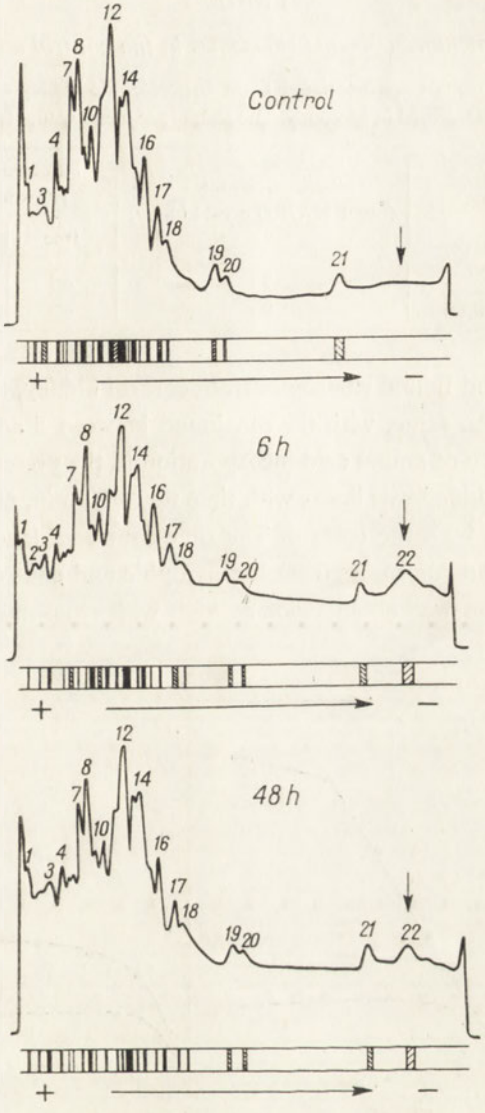


Fig. 1. Polyacrylamide-gel electrophoresis of ribosomal proteins from control and hypertrophic kidney, isolated at the indicated time after unilateral nephrectomy. The electrophoresis was performed at pH 4.5 as described in Methods, 100 μg of ribosomal proteins being applied per gel. The densitometric profiles were recorded at about 465 nm in an ERI-65 Zeiss spectrophotometric scanner.

in the free form (Table 2). In hypertrophic kidney a rise in the total amount of ribosomes by about 20% was observed but there was virtually no increase in the proportion of free ribosomes. This may point to the enhanced activity of free ribosomes in the translation process. This assumption was confirmed by the experiments on the incorporation of [^{14}C]leucine and synthesis of [^{14}C]polyphenylalanine from [^{14}C] Phe-tRNA in cell-free systems.

Table 2

Content of free and membrane-bound ribosomes in the control and hypertrophic kidney

Free and bound ribosomes were isolated from hypertrophic kidney 48 hours after unilateral nephrectomy, as described in Methods. The results are mean values from 5 experiments.

Kidney	Total ribosomes (mg RNA/100 g of kidney)	Percentage of ribosomes	
		free	bound
Control	73	66	34
Hypertrophic	90	62	38

Both with free and bound ribosomes from control kidney, the kinetics of leucine incorporation was the same, with the maximum between 5 and 10 min of incubation (Fig. 2). The rate of amino acid incorporation in the presence of free ribosomes from hypertrophic kidney was linear with time up to 20 min, and was about 4 times higher as compared with the control. The time-course of leucine incorporation in the presence of bound ribosomes from hypertrophic and control kidney was similar but the incorporation was about twice as high with ribosomes from hypertrophic kidney.

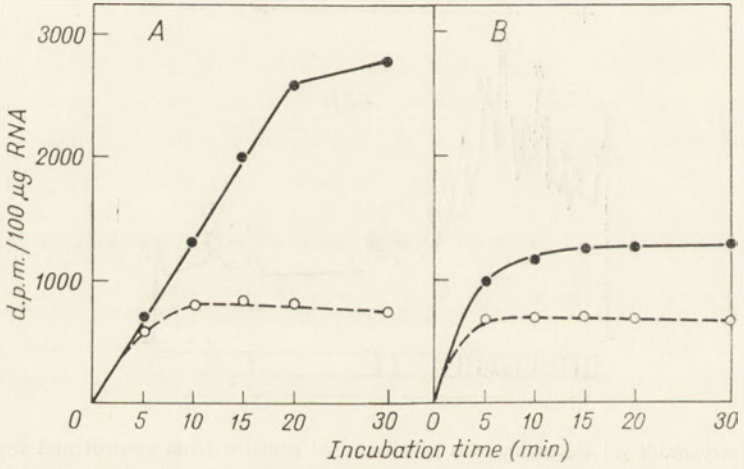


Fig. 2. Incorporation of [^{14}C]leucine in a cell-free system containing: *A*, free and *B*, membrane-bound ribosomes from: ○, control kidney; ●, hypertrophic kidney at 48 h after unilateral nephrectomy. The incubation mixture contained in 0.25 ml: 50 mM-Tris-HCl buffer, pH 7.6; 75 mM-NH_4Cl; 0.5 mM-GTP; 2 mM-ATP; 2 mM-$MgCl_2$; 10 mM-phosphoenolpyruvate; 12 µg of pyruvate kinase; 19 [^{12}C]amino acids, 0.1 mM each; 1 µCi of [^{14}C]leucine (5 µmoles); cytoplasmic factors from control liver (200 µg of protein) and free or bound ribosomes (100 µg of RNA). The incubation was carried out at 37°C and stopped by adding 5 ml of 10% trichloroacetic acid. After 4 h the precipitate was collected on Millipore filter, washed four times with cold trichloroacetic acid, and radioactivity was measured.

Comparison of the activity of the two forms of ribosomes from hypertrophic kidney showed that free ribosomes exhibited an activity about twofold higher than that of bound ribosomes (Table 3).

Table 3

Incorporation of [^{14}C]*leucine by free and membrane-bound kidney ribosomes*

Conditions of the incorporation were the same as described in the legend to Fig. 2, except that the incubation time was 30 min. The ribosomes from hypertrophic kidney were isolated 48 h after unilateral nephrectomy.

Kidney	Ribosomes	Incorporation of [^{14}C]leucine (pmoles/mg of ribosomes)	Increasing factor
Control	free	9.0	—
	bound	9.0	—
Hypertrophic	free	31.3	3.5
	bound	13.6	1.5

Table 4

Synthesis of [^{14}C]*polyphenylalanine from* [^{14}C]*Phe-tRNA by free and membrane-bound ribosomes from control and hypertrophic kidney*

Conditions of the synthesis were the same as described in the legend to Table 1. Ribosomes were isolated from hypertrophic kidney 48 h after unilateral nephrectomy.

Kidney	Ribosomes	Incorporation of [^{14}C]Phe (d. p. m./mg of ribosomes)	Increasing factor
Control	free	2500	—
	bound	400	—
Hypertrophic	free	10200	4
	bound	520	1.3

Higher activity of free ribosomes from hypertrophic kidney was also observed in poly(U)-dependent synthesis of polyphenylalanine from [^{14}C]Phe-tRNA (Table 4). With bound ribosomes from hypertrophic kidney the synthesis was only slightly higher as compared with the control.

DISCUSSION

The experiments presented above confirm our previous observations on the enhanced activity of ribosomes in hypertrophic rat kidney. After unilateral nephrectomy, the activity begins to increase between the 12th and 24th hour, and at 48 h it reaches a value twice as high as that for control ribosomes. These results are at variance with those of Nicholls *et al.* (1975) who observed an increase in ribosome concentration but did not find any changes in the activity of ribosomes obtained from hypertrophic kidney after unilateral nephrectomy, or following administration of folic acid. It seems possible that this lack of change could be due to the high activity of nucleases present in the ribosomal preparations obtained from the kidney

by a procedure not involving the use of inhibitors of these enzymes (Sendecki *et al.*, 1972). There is also the possibility that the ribosomes not washed with KCl were contaminated with cytoplasmic proteins which could interfere with the enhanced activity. In our experiments the ribosomes were isolated in the presence of an inhibitor of nucleases (bentonite) and submitted to extensive washing.

It has been demonstrated by several authors that in regenerating liver and hepatoma cells the ribosomal protein undergoes structural changes. Our earlier observations on ribosomal proteins of hypertrophic kidney also pointed to the occurrence of such changes. In the present work we have found that an additional protein fraction appeared in the ribosomes isolated as early as at 6 h after unilateral nephrectomy, at a time when no changes in ribosome activity could be detected. After 48 h, some changes in the distribution of protein fractions can be seen on the electrophoretic patterns. According to Anderson *et al.* (1975), after partial hepatectomy only the proteins isolated from 40S subunits show changed electrophoretic properties. On the other hand, Scheinbuks *et al.* (1974) demonstrated changes in the relative content of some proteins isolated both from the 40S and 60S subunits of ribosomes from regenerating liver. The character of changes observed in ribosomal proteins has not so far been elucidated. It is not clear whether there occurs a synthesis of new proteins (Rogers, 1973) or whether some proteins normally present in the ribosome become modified (Kabat, 1970; Loeb & Blat, 1970; Stahl *et al.*, 1972; Liew & Gornall, 1973; Eli & Wool, 1973; Gressner & Wool, 1974).

Animal cells are known to contain two forms of ribosomes occurring in the cytoplasm: free and membrane-bound. The ratio of these two forms in various animal cells was found to differ. The cells which produce proteins for export, e.g. liver cells, have a greater amount (60 - 70%) of bound ribosomes (Webb *et al.*, 1965; Takagi & Ogata, 1968, 1971) whereas the cells producing proteins for their own needs, e.g. kidney (Blobel & Potter, 1967), brain or HeLa cells, contain more of free ribosomes (Andrew & Tata, 1971).

Assuming that the two forms of ribosomes present in the cytoplasm are involved in the synthesis of different kinds of protein, it could be expected that, during compensatory hypertrophy of the kidney, the proportion of free ribosomes would be increased.

Although in hypertrophic kidney the content of ribosomes was increased, a very small, if any, change in the proportion of free and bound ribosomes was observed. Similar results were reported by Priestley & Malt (1969) for hypertrophic kidney and by Zweig & Grisham (1971) for regenerating liver. However, although in the hypertrophic kidney there was no increase in the proportion of free ribosomes, their activity in [^{14}C]leucine incorporation and polyphenylalanine synthesis was considerably increased. Since the activity of bound ribosomes was enhanced but slightly, it can be assumed that the activity of free ribosomes is one of the factors responsible for the increased translation process.

The presented results permit to suppose that the increase in ribosome activity observed during compensatory hypertrophy of the kidney concerns mainly the form of free ribosomes which are the site of synthesis of intracellular proteins.

REFERENCES

Anderson, W.M., Grundholm, A. & Sells, B.H. (1975) *Biochem. Biophys. Res. Commun.*, **62**, 669 - 676.

Andrew, T.M. & Tata, J.R. (1971) *Biochem. J.*, **121**, 683 - 694.

Blobel, G. & Potter, V.R. (1967) *J. Mol. Biol.*, **26**, 279 - 292.

Campbell, P.N. & Lawford, G.R. (1967) in *Structure and Function of the Endoplasmatic Reticulum* (ed. F.C. Gran) Academic Press, London - New York.

Campbell, P.N. & Sargent, J.R. (1967) in *Techniques in Protein Synthesis* (eds. P.N. Campbell & J.R. Sargent) vol. 1, pp. 51 - 63. Academic Press, London - New York.

Campbell, P.N. & Sargent, J.R. (1969) in *Techniques in Protein Synthesis* (eds. P.N. Campbell & J.R. Sargent) vol. 1, pp. 301 - 302. Academic Press, London - New York.

Eli, C. & Wool, I.G. (1973) *J. Biol. Chem.*, **248**, 5130 - 5136.

Gesteland, R.F. & Staehelin, T. (1967) *J. Mol. Biol.*, **24**, 149 - 155.

Glazer, R.I. & Sartorelli, A.C. (1972) *Biochem. Biophys. Res. Commun.*, **46**, 1418 - 1424.

Gressner, A.M. & Wool, I.G. (1974) *J. Biol. Chem.*, **249**, 6917 - 6925.

Halliburton, I.W. (1969) in *Compensatory Renal Hypertrophy* (eds. W.W. Nowiński & R.J. Goss) pp. 101 - 130. Academic Press, New York.

Hill, J.M. (1975) *J. Cell. Biol.*, **64**, 260 - 265.

Hill, J.M. & Malamud, D. (1974) *FEBS Lett.*, **46**, 308 - 311.

Hill, J.M., Ab, G. & Malt, R.A. (1974) *Biochem. J.*, **144**, 447 - 453.

Kabat, D. (1970) *Biochemistry*, **9**, 4160 - 4175.

Liew, C.C. & Gornall, A.G. (1973) *J. Biol. Chem.*, **248**, 977 - 983.

Loeb, J.E. & Blat, C. (1970) *FEBS Lett.*, **10**, 105 - 108.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.

Malt, R.A. & Lemaitre, D.A. (1968) *Amer. J. Physiol.*, **214**, 1041 - 1047.

Moldave, K. (1963) in *Methods in Enzymology* (eds. S.P. Colowick & N.O. Kaplan) vol. 6, pp. 757 - 761. Academic Press, New York - London.

Moldave, K. & Skogerson, L. (1967) in *Methods in Enzymology* (eds. L. Grossman & K. Moldave) vol. 12A, pp. 478 - 481. Academic Press, New York - London.

Moldave, K., Gałasiński W. & Rao, P. (1971) in: *Methods in Enzymology* (eds. L. Grossman & K. Moldave) vol. 20, pp. 337 - 348. Academic Press, New York - London.

Nicholls, D.M., Chan, M. & Girgis, G.R. (1975) *Development Biology*, in press.

Nowiński, W.W & Goss, R.J. (1969) *Compensatory Renal Hypertrophy*. Academic Press, New York.

Priestley, G.C. & Malt, R.A. (1969) *J. Cell. Biol.*, **41**, 886 - 893.

Rogers, A. (1973) *Biochim. Biophys. Acta*, **294**, 292 - 296.

Scheinbuks, J., Sypherd, P.S. & Moldave, K. (1974) *Biochem. Biophys. Res. Commun.*, **61**, 322 - 328.

Sendecki, W., Ombach, M. & Kwiatkowska-Patzer, B. (1972) *Bull. Acad. Polon. Sci., ser. sci. biol.*, **20**, 273 - 278.

Sendecki, W., Kuliszewski, M. & Patzer, J. (1973a) *Acta Biochim. Polon.*, **20**, 63 - 71.

Sendecki, W., Patzer, J. & Kuliszewski, M. (1973b) *Acta Biochim. Polon.*, **20**, 383 - 393.

Spitnik-Elson, P. (1965) *Biochem. Biophys. Res. Commun.*, **18**, 557 - 562.

Stahl, J., Welfle, H. & Bielka, H. (1972) *FEBS Lett.*, **26**, 233 - 236.

Takagi, M. & Ogata, K. (1968) *Biochem. Biophys. Res. Commun.*, **33**, 55 - 60.

Takagi, M. & Ogata, K. (1971) *Biochem. Biophys. Res. Commun.*, **42**, 125 - 131.

Trachewsky, D., Nandi Majumdar, A.P. & Congote, L.F. (1972) *Eur. J. Biochem.*, **26**, 543 - 552.

Webb, T.E., Blobel, G., Potter, V.R. & Morris, H.P. (1965) *Cancer Res.*, **25**, 1219 - 1224.

Willems, M., Musilova, H.A. & Malt, R.A. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 1189 - 1194.

Zweig, M. & Grisham, J.W. (1971) *Biochim. Biophys. Acta*, **246**, 70 - 80.

WPŁYW JEDNOSTRONNEJ NEFREKTOMII NA AKTYWNOŚĆ RYBOSOMÓW W POZOSTAŁEJ NERCE

Streszczenie

1. Rybosomy izolowano z przerastających nerek w różnych odstępach czasu po jednostronnej nefrektomii. Aktywność rybosomów w inkorporacji [^{14}C]leucyny i syntezie polifenyloalaniny z [^{14}C]Phe-tRNA była zwiększona po 24 godzinach, natomiast zmiany w składzie białkowym rybosomów stwierdzono już w 6 godzin po operacji.

2. W nerce przerastającej zwiększa się ogólna ilość rybosomów, jednak stosunek rybosomów wolnych i związanych z endoplazmatycznym retikulum pozostaje praktycznie niezmieniony. Natomiast aktywność wolnych rybosomów jest dwukrotnie większa niż związanych.

3. Na podstawie otrzymanych wyników można przyjąć, że frakcja wolnych rybosomów jest jednym z czynników odpowiedzialnych za zwiększoną syntezę białka w nerce przerastającej.

Received 16 August, 1975.

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 1

IRENA PASTUSZAK and M. SZYMONA

PURIFICATION AND PROPERTIES OF L-ASPARAGINASE FROM *MYCOBACTERIUM PHLEI*

Department of Biochemistry, Institute of Basic Chemical Sciences, Medical School of Lublin, Lubartowska 85, 20-123 Lublin, Poland

1. L-Asparaginase from *M. phlei* was purified about 170-fold with an 11% yield. The purification procedure consisted of: fractionation with ammonium sulphate; adsorption of contaminating proteins on calcium phosphate gel; chromatography on Sephadex G-150 and DEAE-cellulose. The specific activity of the final preparation was 32.6 i.u./mg protein.

2. Molecular weight of the enzyme as determined by Sephadex G-100 filtration amounted to 126 000. Optimum pH was 8.8 - 9.2. The enzyme did not hydrolyse L-glutamine over the pH range 4 - 9, and was inhibited by D-asparagine. The apparent Michaelis constant for L-asparagine was 0.7 mM; energy of activation, 9800 cal/mole.

3. On polyacrylamide-gel electrophoresis the final preparation revealed two protein bands, one of which was coincident with the enzyme activity.

Preliminary information on L-asparaginase in mycobacteria was reported by Kirchheimer & Whittaker (1954), Halpern & Grossowicz (1957) and Ott (1960). In 1968 Jayaram *et al.* partially purified L-asparaginase from *M. tuberculosis* and supplied evidence for the presence of two enzymic forms of this enzyme, differing in pH optimum, thermostability and sensitivity to various inhibitors. One of the isoenzymic forms of *M. tuberculosis* showed oncostatic properties (Reddy *et al.*, 1969). Recently, Soru *et al.* (1972) described a homogeneous preparation of L-asparaginase from *Mycobacterium bovis* BCG strain. Despite 100-fold purification, the enzyme preparation exhibited rather low specific activity of about 5 i.u./mg protein (pH 8.6, 37°C).

In our studies, *M. phlei* was found to be a relatively rich source of L-asparaginase as compared with other mycobacteria. When cultivated on Sauton's medium, the strain proved capable of producing L-asparaginase yielding up to 6 i.u./g wet weight of bacteria after 3 weeks of incubation (Pastuszak & Szymona, 1975). The present work is concerned with purification and characterization of this enzyme.

MATERIAL AND METHODS

Chemicals. L-Asparagine and D-asparagine were obtained from Reanal (Budapest, Hungary). L-Glutamine was an imported product supplied by POCh (Gliwice, Poland). Tris-hydroxymethylaminomethane p.a. was from Loba-Chemie (Wien, Fischamend, Austria). DEAE-cellulose DE 52 was purchased from Whatman Biochemicals Ltd. (Maidstone, Kent, England). Blue Dextran 2000, Sephadex G-100 and Sephadex G-150 superfine were obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol 20 000 and acrylamide were from R.C.B. (Bruxelles, Belgium). 1,4-Tetramethylenediamine was obtained from Fluka A.G. (Buchs S.G., Switzerland). *N,N'*-bis-acrylamide was purchased from B.D.H. Chemicals Ltd. (Poole, Dorset, England). Amido Black 10 B was from Chemapol (Czechoslovakia). Bovine serum albumin and pancreatic deoxyribonuclease (salt-free, lyoph., 450 units/mg) were from Koch-Light (Colnbrook, Bucks., England). Cytochrome *c* was from Sigma Chem. Co. (St. Louis, Mo., U.S.A.), myoglobin from Calbiochem (Los Angeles, Calif., U.S.A.), trypsin from Merck (Darmstadt, G.F.R.). Haemoglobin was from Downs Development Chemicals (Ruislip, England). Aldolase (rabbit muscle) was from Boehringer und Soehne GmbH (Mannheim, G.F.R.). Calcium phosphate gel was prepared according to Keilin & Hartree (Colowick, 1955). Other chemicals were commercial products supplied by POCh (Gliwice, Poland).

Microorganism and cultivation. The strain of *Mycobacterium phlei* was maintained in this laboratory for several years by mass transfers on Lowenstein slopes. For experiments, the organism was grown on 200 ml portions of Sauton's medium in 1 litre Roux flasks. After 3 weeks of static growth at 37°C, the cells were harvested by filtration and washed with water at room temperature. The yield of cells amounted up to 40 g of wet weight per litre of the medium. The washed bacteria were stored in the frozen state (—15°C) until needed.

L-Asparaginase assay. The incubation mixture contained in a total volume of 2.0 ml: 20 μmoles of L-asparagine, 375 μmoles of Tris-HCl buffer, pH 9.0 and enzyme (up to 0.4 i.u.). If not otherwise stated, the reaction was run at 37°C for 5-20 min depending on enzyme activity. After incubation, 0.25-ml aliquots of the reaction mixture were transferred to the outer part of Conway vessels containing 1 ml of saturated solution of K_2CO_3. The inner compartment contained 1.5 ml of 0.1 M-HCl. Following 2.5-h diffusion, a 1.0-ml portion of the inner solution was mixed in a test tube with 1.0 ml of water, 5.0 ml of Nessler's reagent and 1.0 ml of 10 M-NaOH. The absorbance was read at 420 nm using Spekol spectrocolorimeter. One unit of activity was defined as the amount of enzyme which hydrolysed 1 μmole of L-asparagine in one minute at 37°C.

In some cases, the activity was determined on the basis of aspartic acid formation. Paper electrophoresis was used to separate the product from L-asparagine.

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Disc electrophoresis was performed after Davis (1964) in a 7.5% acrylamide gel, pH 8.9. Before use, acrylamide and *N,N'*-bisacrylamide were recrystallized from

chloroform and acetone, respectively (Loening, 1967). The electrophoresis was run for 3 h at 3 mA per gel (5 × 0.5 cm) at 4°C. Protein was stained with 1% Amido Black in 7% acetic acid for 1 h. The gels were destained electrophoretically (10 mA/gel) with 7% acetic acid.

Molecular weight was determined by gel filtration on Sephadex G-100 according to Andrews (1964), using 0.01 M-phosphate buffer, pH 7.1, for equilibration. The void volume (V_0) of a 80 × 1.5 cm column as established with Blue Dextran was 44 ml. The column was calibrated with cytochrome *c* (12 400), myoglobin (17 800), haemoglobin (64 000), trypsin (23 800) and aldolase (149 000). The elution volumes (V_e) of the first three proteins were determined both at 450 nm and with the Folin reagent, that of trypsin by the Kunitz method (Laskowski, 1955), and of aldolase according to Sibley & Lehninger (1949).

RESULTS AND DISCUSSION

Purification of the enzyme

All steps of the procedure were carried out at 0 - 4°C.

1. Preparation of crude extract. Sixty grams of cells (wet weight) were suspended in 300 ml of cold 5 mM-phosphate buffer, pH 7.2, and disrupted in 30-ml portions using a MSE 100 W ultrasonic vibrator, 24 kc/s (2 × 5 min). The homogenate was centrifuged for 40 min at 12 000 rev./min in the Janetzki refrigerated centrifuge K70 and the resulting extract was incubated for 1.5 h at room temperature with 1 mg of DNase and 100 µmoles of $MgCl_2$ per 100 ml.

2. Fractionation with ammonium sulphate. To 280 ml of the extract, solid ammonium sulphate was added slowly to 0.35 saturation and the mixture kept for 30 min in ice-cold water. After centrifugation (12 000 rev./min, 40 min), the pellet was discarded and the supernatant brought to 0.75 saturation with solid ammonium sulphate. The 0.35 - 0.75 sat. ammonium sulphate precipitate (fraction AS-2) was dissolved in 0.01 M-phosphate buffer, pH 6 (1/5 of the initial extract volume). The solution was then dialysed against the same buffer (2 × 2 litres) until disappearance of SO_4^{2-} ions.

3. Negative adsorption on calcium phosphate gel. To the dialysed AS-2 fraction, diluted to contain 6 mg protein/ml, calcium phosphate gel was added with constant stirring (3 mg of dry gel/mg protein). After 10 min, the precipitate was removed by centrifugation (3000 rev./min) and the enzyme protein was salted out with solid ammonium sulphate at 0.75 saturation. The final precipitate was dissolved in 5 ml of 0.01 M-phosphate buffer, pH 6.5.

The above conditions of negative adsorption were found optimal in a series of determinations.

4. Molecular sieve chromatography. The solution from the previous step was applied to the Sephadex G-150 column (2.6 × 85 cm) and eluted with 0.01 M-phosphate buffer, pH 6.5, at a flow rate of 8 - 10 ml/h. Fractions of 6 ml were collected.

5. Chromatography on DEAE-cellulose. The pooled Sephadex G-150 fractions (48 ml) were applied to a DEAE-cellulose column (10 × 1.5 cm) equilibrated with 0.01 M-phosphate buffer, pH 7.2, containing 1 mM-2-mercaptoethanol and 1 mM-EDTA, and eluted stepwise with increasing concentration of NaCl solutions (0.05, 0.10, 0.15 M in the same buffer) at a flow rate of 45 ml/h. The enzyme emerged at the NaCl concentration of 0.15 M.

The over-all results of the isolation procedure are summarized in Table 1. As one can see, the final solution exhibits a specific activity of 32.6 i. u/mg protein, which indicates a 171-fold purification with 11% recovery. The efficiency of the method adopted was tested by polyacrylamide-gel electrophoresis. The final preparation revealed two well separated protein bands, one of which could be identified as L-asparaginase (Fig. 1). The enzyme was localized in the upper band by incubating the successive gel discs in the reaction mixtures for the enzyme assay (see Methods), and the aspartate formed in the reaction was revealed by paper electrophoresis.

Table 1

Purification of L-*asparaginase from M. phlei grown for 3 weeks on Sauton's medium*

The enzyme activity is expressed in μmoles of NH_3 liberated per 1 min (37° C) in standard reaction mixture. For details see the text

Purification step	Activity		Purification factor	Yield (%)
	total	per mg protein		
Cell-free extract	387	0.19	1	100
Ppt. at 0.35 - 0.75 $(NH_4)_2SO_4$ sat.	369	0.39	2	95
Calcium phosphate gel supernatant	198	2.47	13	51
Sephadex G-150 eluate	101	14.0	74	26
DEAE-cellulose eluate (pooled)	39	32.6	171	11

The relatively high degree of purification obtained is mainly due to the calcium phosphate gel treatment and Sephadex G-150 chromatography. The L-asparaginase preparation from *M. tuberculosis* $H_{37}Rv$ exhibiting an about 2.5-fold lower specific activity, was obtained by Jayaram *et al.* (1968) by ammonium sulphate fractionation, calcium phosphate gel treatment and DEAE-cellulose chromatography. Soru *et al.* (1972) purified the enzyme from *M. tuberculosis bovis* using negative adsorption on calcium phosphate gel, DEAE-Sephadex A-50 chromatography, Bio-Gel P-200 filtration and crystallization. The final enzyme preparation proved to be homogeneous on polyacrylamide-gel electrophoresis and immunodiffusion. However, its specific activity was about 1/6 that of the *M. phlei* enzyme preparation.

Properties of the enzyme

If not otherwise stated, the properties of the *M. phlei* L-asparaginase were studied using the calcium phosphate gel supernatant. This preparation was more stable than either the Sephadex or DEAE-cellulose eluates.

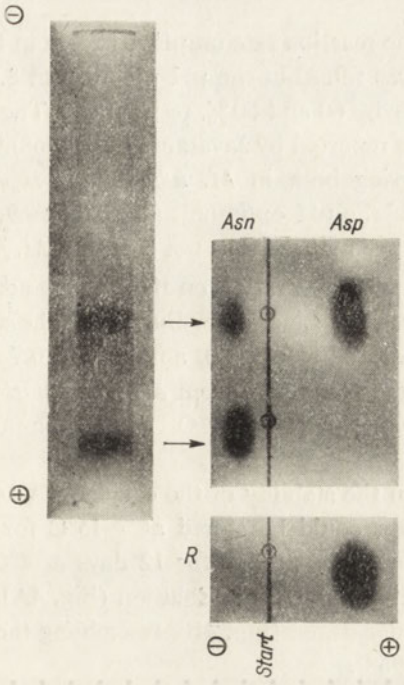


Fig. 1. Polyacrylamide-gel electrophoresis of the purified L-asparaginase from *M. phlei*. The enzyme (40 μg protein), concentrated by dialysis against solid polyethylene glycol 20 000, was analysed as described in Methods. One of the two parallelly run electrophoretogram gels was stained and the other cut into discs, in which the enzyme activity was determined after incubation in 0.5 ml of the standard reaction mixture for 1.5 h. Then 10-μl aliquots were submitted to paper electrophoresis on Whatman no. 1 filter paper (42 × 25 cm) in pyridine - glacial acetic acid - water (3:10:487, by vol.) for 1 h at 600 V. The amino acids were detected with 0.1% ninhydrin in acetone. *R*, reference spot of aspartic acid.

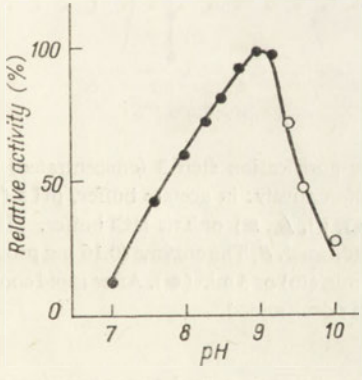


Fig. 2

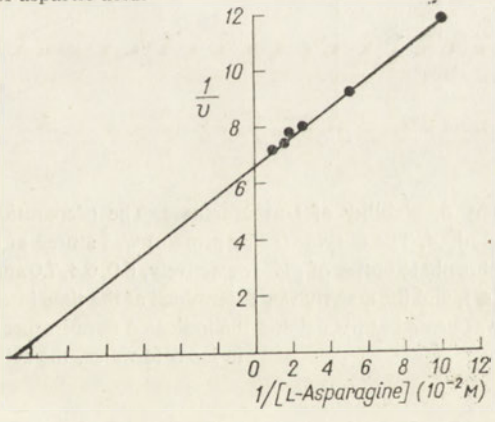


Fig. 3

Fig. 2. Effect of pH on L-asparaginase activity. The enzyme (0.1 mg protein) following negative adsorption on calcium phosphate gel (purification step 3) was used. Incubation conditions as described in Methods. The standard reaction mixture with: ●, Tris-HCl and ○, glycine-NaOH buffers was applied.

Fig. 3. Effect of substrate concentration on L-asparaginase activity. The enzyme (0.16 mg protein) after purification step 3 was used. Incubation conditions as described in Methods.

The dependence of the reaction rate on pH is shown in Fig. 2. As it can be seen, the maximum activity was found in the pH range from 8.8 to 9.2. At pH 7.5 and 9.5 the activity was lower by 60 and 50%, respectively. The optimum pH is in good agreement with the value reported by Jayaram *et al.* (1968) for one of the two forms of L-asparaginase occurring both in *M. tuberculosis* $H_{37}Ra$ and *M. tuberculosis* $H_{37}Rv$. Another peak of the pH optimum, observed at 9.6 with the extract from the avirulent strain by these authors, was not found in *M. phlei*.

The effect of substrate concentration on the enzyme activity is shown in Fig. 3. As can be calculated from the Lineweaver-Burk plot, the apparent Michaelis constant for the enzyme measured at pH 9.0, amounts to 0.7 mM. This value is much lower than that for the L-asparaginase from *M. tuberculosis* $H_{37}Ra$ (2.0 mM), but is in agreement with the value reported by Ott (1960) for the *M. smegmatis* enzyme (0.74 mM).

In the experiments on the stability of the enzyme it was fonnd that the calcium phosphate gel supernatant could be stored at —15°C for one month without an appreciable loss of activity. On storage for 12 days at 4°C, the activity decreased in dependence on the pH of the enzyme solution (Fig. 4A). At higher temperature, the enzyme was rapidly denatured (Fig. 4B), resembling the "pH 9.0" enzymes studied by Jayaram *et al.* (1968).

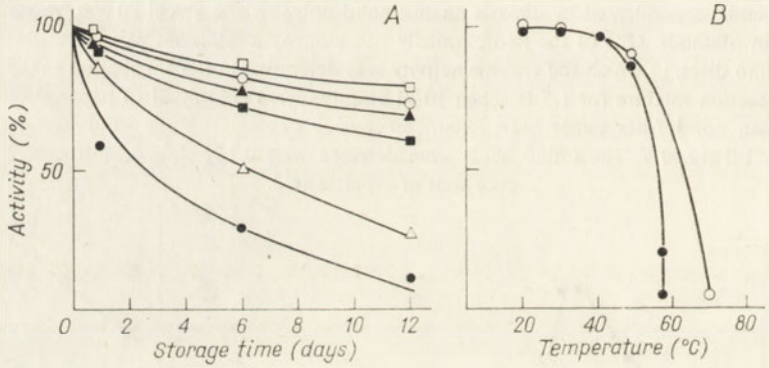


Fig. 4. Stability of L-asparaginase. The preparation after purification step 3 (concentrated) was used. *A*, The enzyme (82 μg protein) was stored at 4°C, alternatively: in acetate buffer, pH 5 (△); phosphate buffer of pH, respectively, 6.0, 6.4, 7.0 and 7.9 (○, □, ▲, ■) or Tris-HCl buffer, pH 9.0 (●), and the activity was determined at the time intervals indicated. *B*, The enzyme (0.16 mg protein) was heated at pH 6.5 and the indicated temperature for: 1 min (○) or 3 min (●). After rapid cooling in ice-cold water, the activity was determined.

In the subsequent experiments the effect of L-glutamine and D-asparagine was tested. The reaction mixtures contained 350 μmoles of the buffer over the pH range from 4 to 9, 20 μmoles of L-glutamine or D-asparagine and 45 μg of protein (0.3 ml of the Sephadex G-150 eluate) in a total volume of 2.0 ml; after 30-min incubation, the ammonia formed was determined as described in Methods. L-Glutamine was not hydrolysed in any of the seven samples at the pH range studied. D-Asparagine was

only effective at pH 9 but at a very low rate, which constituted less than 5% of that shown by the L-isomer. However, in the presence of the D-isomer added at equimolar concentration, the hydrolysis of L-asparagine was inhibited by about 60%. These data, showing high specificity of the L-asparaginase from *M. phlei* toward asparagine and the inhibitory effect of D-asparagine, are in accordance with the earlier findings of Halpern & Grossowicz (1957) and Ott (1960).

The effect of temperature on the L-asparaginase is shown in Fig. 5A. The energy of activation was calculated to be 9800 cal/mole (Fig. 5B). For the *E. coli* enzyme the corresponding value was 7440 cal/mole (Mori *et al.*, 1973).

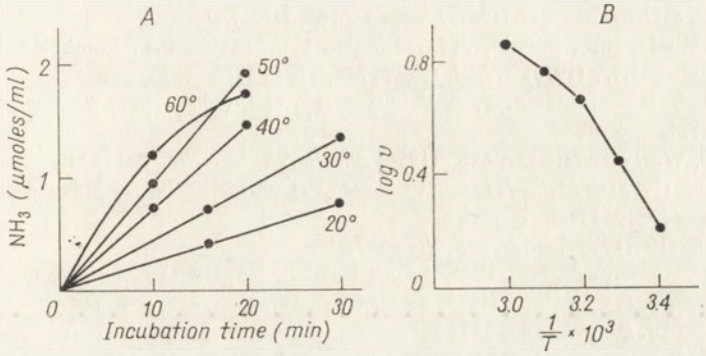


Fig. 5. Effect of temperature on L-asparaginase activity. The enzyme (80 μg protein) after purification step 3 was used. Prior to the addition of the enzyme, reaction mixtures were preheated for 2 min at the temperature indicated. *A*, Time-course of the reaction; *B*, Arrhenius plot.

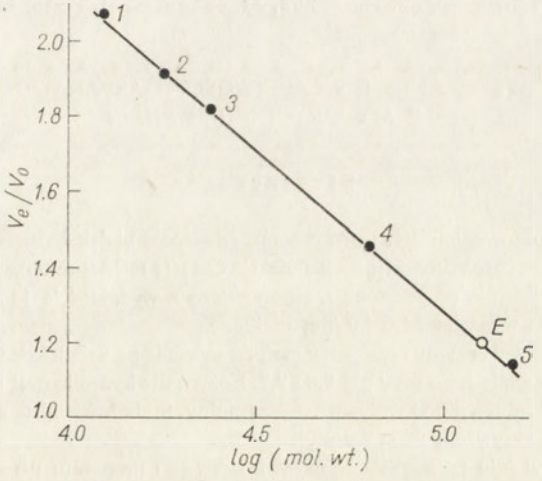


Fig. 6. Determination of molecular weight of L-asparaginase. The concentrated enzyme (0.95 mg protein) after purification step 4 was applied to a Sephadex G-100 column (80 × 1.5 cm) and eluted with 0.01 M-phosphate buffer, pH 7.1. Fractions of 2 ml were collected at a flow rate of 20 ml/h. The column was calibrated with: *1*, cytochrome *c* (12 400); *2*, myoglobin (17 800); *3*, trypsin (23 800); *4*, haemoglobin (64 000) and *5*, aldolase (149 000). *E*, L-asparaginase (mol. wt. 126 000).

The molecular weight of the mycobacterial enzyme as estimated by Sephadex G-100 gel filtration was found to be 126 000 (Fig. 6), which corresponds to the values reported for L-asparaginases of *Proteus vulgaris* (Tosa *et al.*, 1973) and *E. coli* (Ho *et al.*, 1970).

REFERENCES

Andrews, P. (1964) *Biochem. J.*, **91**, 222 - 226.

Colowick, S.P. (1955) in *Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, eds.) vol. 1, pp. 90 - 98. Academic Press, New York.

Davis, G. (1964) *Ann. N. Y. Acad. Sci.*, **121**, 404 - 427.

Halpern, Y.S. & Grossowicz, N. (1957) *Biochem. J.*, **65**, 716 - 720.

Ho, P.P.K., Milkin, E.B., Bobbitt, J. L., Grinnan, E.L., Burck, P.J., Frank, B.H., Boeck, V.D. & Squires, R.W. (1970) *J. Biol. Chem.*, **245**, 3708 - 3715.

Jayaram, H.N., Ramakrishnan, T, & Vaidyanathan, C.S. (1968) *Arch. Biochem. Biophys.*, **126**, 165 - 174.

Kirchheimer, W.F. & Whittaker, C.K. (1954) *Am. Rev. Tuberc.*, **70**, 920 - 921.

Laskowski, M. (1955) in *Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, eds.) vol. 2, pp. 8-26. Academic Press, New York.

Loening, U.E. (1967) *Biochem. J.*, **102**, 251 - 257.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.

Mori, T., Tosa, T. & Chibata, I. (1973) *Biochim. Biophys. Acta*, **321**, 653 - 661.

Ott, J.L. (1960) *J. Bacteriol.*, **80**, 355 - 361.

Pastuszak, I. & Szymona, M. (1975) *Acta Microbiol. Polon.* **8**, 131 - 139.

Reddy, V.V.S., Jayaram, H.N., Sirsi, M. & Ramakrishnan, T. (1969) *Arch. Biochem. Biophys.*, **132**, 262 - 267.

Sibley, J.A. & Lehninger, A.L. (1949) *J. Biol. Chem.*, **177**, 859 - 872.

Soru, E., Teodorescu, M., Zaharia, O., Szabados, J. & Rudescu, K. (1972) *Can. J. Biochem.*, **50**, 1149 - 1157.

Tosa, T., Sano, R., Yamamoto, K., Nakamura, M. & Chibata, I. (1973) *Biochemistry*, **12**, 1075 - 1079.

OCZYSZCZANIE I WŁAŚCIWOŚCI L-ASPARAGINAZY Z *MYCOBACTERIUM PHLEI*

Streszczenie

1. Stosując frakcjonowanie siarczanem amonu, adsorpcję białek balastowych na żelu fosforanowo-wapniowym oraz chromatografię na Sefadeksie G-150 i DEAE-celulozie otrzymano z ekstraktu *M. phlei* ok. 170-krotnie oczyszczoną L-asparaginazę z wydajnością 11%. Aktywność swoista końcowego preparatu wynosiła 32,6 i.u./mg białka.

2. Ciężar cząsteczkowy enzymu oznaczony metodą sączenia na Sefadeksie G-100 wynosił 126 000. Optimum aktywności znaleziono przy pH 8,8 - 9,2. Enzym nie hydrolizował L-glutaminy w zakresie pH od 4 do 9 i ulegał inhibicji pod wpływem D-asparaginy. Stała Michaelisa dla L-asparaginy wynosiła 0,7 mM; energia aktywacji, 9800 cal/mol.

3. Elektroforeza w żelu poliakrylamidowym końcowego preparatu ujawniła dwa pasma białkowe, z których jedno odpowiadało aktywności enzymatycznej.

Received 30 August, 1975.

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 1

MAŁGORZATA HANAUSEK-WAŁASZEK, JADWIGA MICHALSKA
and M. CHORĄŻY

SOME FEATURES OF DNA-rRNA HYBRIDS IN THE RAT*

Department of Tumour Biology, Institute of Oncology,
ul. Armii Czerwonej 15; 44-100 Gliwice, Poland

1. Sequences complementary to rRNA in rat DNA amount to 0.04% which corresponds to about 300 rRNA genes per haploid genome.

2. Purified DNA-rRNA hybrids had a DNA : rRNA weight ratio of about 1.1 : 1. Their melting temperature estimated on hydroxyapatite column was 75 - 80°C. In CsCl gradients hybrids banded at 1.728 g/cm^3 as compared to 1.699 g/cm^3 for bulk DNA.

3. Length distribution histogram of DNA-rRNA hybrid molecules visualized by electron microscopy revealed that most of them possessed a length of 0.2 - 0.5 μm which corresponds to 4×10^5 - 1×10^6 daltons, i.e. 670 - 1600 nucleotide pairs.

4. DNA sequences complementary to rRNA are localized on the heavy shoulder of the main band at a CsCl density of 1.709 g/cm^3, and in a DNA fraction which reassociates at a $C_0t = 10^{-1}$ - 10^2.

The anatomy of genes coding for ribosomal RNA has been described in great detail for *Xenopus laevis* (Birnstiel *et al.*, 1966, 1968; Brown & Weber, 1968a,b; Dawid *et al.*, 1970; Reeder & Brown, 1970; Brown & Blacker, 1972; Wellauer & Dawid, 1974; Wellauer *et al.*, 1974), *Triturus viridescens* (Miller & Beatty, 1969a,b; Scheer *et al.*, 1975), *Drosophila melanogaster* (Ritossa & Spiegelman, 1965; Quagliarotti & Ritossa, 1968; Tartoff & Perry, 1970), and *Tetrahymena pyriformis* (Gall, 1974; Yao *et al.*, 1974). The knowledge about ribosomal genes in other species is generally limited to the estimation of the number of DNA copies complementary to rRNA.

Simultaneously with our previous studies on the rat genome (Szala *et al.*, 1971; Szala & Chorąży, 1972) we have undertaken a series of experiments concerning DNA complementary to rRNA in this species; the results of this study are reported below.

* This work was supported by the Polish Academy of Sciences within the project 09.3.1.

MATERIALS AND METHODS

Reagents: [2-^{14}C]Thymidine, code no. CFA. 219 (spec. act. 35.9 mCi/mmole), [5-^{3}H]uridine, code no. 178 (spec. act. 24 Ci/mmole) were products of The Radiochemical Centre (Amersham, Bucks., England). [^{32}P]Orthophosphate, code no. RP-13 (spec. act. 20 mCi/mmole) was from The Institute for Nuclear Research (Świerk, Poland).

Sodium dodecyl sulphate[1] was from B.D.H. Chemicals Ltd. (Poole, Dorset, England). Tris, cytochrome *c*, and ribonuclease A were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), triisopropyl naphthalenesulphonate sodium salt, and polyvinyl sulphate sodium salt were purchased at Serva Feinbiochemica (Heidelberg, G.F.R.), acrylamide, *N,N*-methylene-bis-acrylamide, and 1,4-*N,N,N',N'*-tetramethylenediamine, 2-mercaptoethanol, PPO, POPOP, ethyleneglycol and cetyltrimethylamine bromide were supplied by Koch-Light Lab. Ltd. (Colnbrook, Bucks, England).

Agarose was from International Enzymes Ltd. (Windsor, Berkshire, England). Whatman DEAE-cellulose was from W.R. Balston, Ltd. (Maidstone, Kent, England) millipore filters (HAWP 13) from Millipore Corporation (Bedford, Mass., U.S.A.). Sephadex G-50 and G-100 from Pharmacia (Uppsala, Sweden), Eagle's medium from Difco Lab. (Detroit, Mich., U.S.A.). Calf serum was supplied by the Laboratory of Sera and Vaccines (Lublin, Poland).

Pancreatic deoxyribonuclease (DNase) electrophoretically purified was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.), and ribonuclease T_1 from Sankyo Co. Ltd. (Tokyo, Japan). Pronase B grade was supplied by Calbiochem (Los Angeles, Calif., U.S.A.) and toluidine blue by E. Merck (Darmstadt, G.F.R.). All other reagents were analytical grade and were purchased at POCh (Gliwice, Poland).

Materials. DNA and rRNA were isolated from livers of Wistar rats. Animals were fasted for 16 - 20 h and killed under light ether anaestesia. Labelled DNA and rRNA was obtained from rat kidney fibroblasts transformed with SV-40 virus and grown in culture on Eagle's medium with 10% calf serum. For control experiments DNA and rRNA was isolated from *E. coli*, strain K12.

Isolation of DNA. Rat livers were perfused with 0.15 M-NaCl and placed into cold (0°C) 0.1 M-NaCl containing 0.04% SDS, cut into small fragments, and homogenized in a glass-teflon homogenizer. DNA was isolated according to the method of Savitsky & Stand (1966) from a crude nuclear pellet obtained by centrifugation of the homogenate at 1000 *g*. DNA was purified by digestion with RNase A (20 μg/ml, in 0.1 × SSC, 30 min, at 37°C) and pronase B (200 μg/ml, in 0.1 × SSC, 2 h, at 37°C). The DNA preparation was then deproteinized with chloroform - isoamyl alcohol (24:1, v/v).

[1] Abbreviations: SDS, sodium dodecyl sulphate; CTAB, cetyltrimethylamine bromide; SSC, 0.15 M-NaCl - 0.015 M-sodium citrate; HAP, hydroxyapatite; BD-cellulose, benzoylated DEAE-cellulose; PB, sodium phosphate buffer, pH 6.8.

Labelled DNA was obtained by the same procedure from a culture of fibroblasts supplemented for 72 h with [^{14}C]thymidine at a concentration of 5 - 10 μCi/ml of Eagle's medium.

Isolation of rRNA. Perfused livers were put into a cold solution composed of 25 mM-Tris-HCl, pH 7.6, 25 mM-KCl, 5 mM-$MgCl_2$, and 250 mM-saccharose. Polyribosomes were obtained by the method of Palacios *et al.* (1972), and rRNA was isolated from polyribosomes by the phenol-cresol method of Kirby (1965). The rRNA preparation was purified by precipitation with CTAB and deproteinized with pronase B (200 μg/ml in 20 mM-NaCl, 2 h, 37°C). After a second phenol extraction, rRNA was precipitated with 2 M-LiCl and then purified by gel electrophoresis.

Labelled rRNA was isolated by the same procedure from rat embryo fibroblasts grown in culture in Eagle's medium supplemented for 24 - 48 h with [^{3}H]uridine (10 μCi/ml) or for 24 h with [^{32}P]orthophosphate (20 μCi/ml). Specific activities of [^{3}H]rRNA ranged from 30 000 to 180 000 c.p.m./μg RNA, and of [^{32}P]rRNA from 10 000 to 53 000 c.p.m./μg.

The base composition of rRNA was determined as described by Katz & Comb (1963) either by spectrophotometric or radioactivity measurements of the four 2′,3′-ribonucleotides formed on alkaline hydrolysis of rRNA.

Polyacrylamide-gel electrophoresis. Acrylamide was recrystallized from chloroform, and bis-acrylamide from acetone according to the procedure of Loening (1967) and 2.2 - 2.4% gels with 0.5% agarose were used. Gel slabs were about 8 cm long and 0.6 cm in diameter, current 3 mA, time 2 h, and the temperature 4°C. After electrophoresis, the gel slabs were fixed in 1 M-acetic acid for 20 min, stained in 0.2% toluidine blue in 1% acetic acid, and destained overnight with 7% acetic acid. Radioactivity of ^{32}P-labelled rRNA in gel slices (1 mm) was counted as Čerenkov radiation. When rRNA was labelled with ^{3}H, gel slices were incubated overnight at 65°C in 0.5 ml of concentrated formic acid and the radioactivity counted in PPO - POPOP - glycol - toluene fluid.

To recover 28S and 18S rRNA from the gel, the segments containing rRNA were placed in plastic tubes closed with dialysis tubing, the tubes were filled with a buffer prepared according to Loening (1967), consisting of: 30 mM-Tris, 30 mM-NaH_2PO_4 and 1 mM-EDTA, pH 7.6, then put into an electrophoretic apparatus and a current of 10 mA at 4°C was applied for 4 - 5 h. After this time, fractions of rRNA were removed from the dialysing sack with a syringe.

DNA-rRNA hybridization on millipore filters. We used the technique of Gillespie & Spiegelman (1965); 10 - 20 μg of thermally denatured DNA was loaded under light vacuum to a filter presoaked in 2 × SSC. The filter was first dried in air, then at 80°C for 2 h, and incubated in the rRNA solution in 2 × SSC at 65°C for a desired time. After hybridization the filter was treated with RNase A (500 μg in 10 ml of 2 × SSC) and T_1 (50 units in 10 ml of 2 × SSC), washed with a large volume of 2 × × SSC, dried at 100°C in vacuum for 2 - 3 h, and radioactivity counted in scintillation liquid. The amount of DNA remaining on the filter was determined after hydrolysis of DNA with 1 M-HCl according to the method of Brown & Weber (1968a).

DNA-rRNA hybridization in solution and isolation of hybrids. DNA at a concentration of 100 μg/ml in 0.02 M-NaCl was denatured for 10 min in boiling water, then treated for 5 min with 0.1 M-NaOH (final concentration). A sample was neutralized with 1 M-HCl, and the final concentration of NaCl was raised up to 0.3 M.

Denatured DNA (100 μg/ml) and [^{3}H]rRNA (50 μg/ml) were dissolved in 2× ×SSC, heated for 5 min at 100°C and incubated at 65°C (Nygaard & Hall, 1964) in tightly closed vials. The mixture was then digested at 37°C for 30 min with nuclease S_1 obtained from takadiastase by the method of Sutton (1971), followed by ribonuclease A and T_1 (50 μg/ml of 0.02 M-NaCl, and 5 units/ml, respectively). DNA-rRNA hybrids were then isolated and purified by hydroxyapatite and benzoylated DEAE-cellulose chromatography.

Hydroxyapatite was prepared according to the method of Miyazawa & Thomas (1965). The hybridization mixture after the enzymatic treatment was dialysed overnight against 0.035 M-Na-phosphate buffer, pH 6.8, loaded on a 3×2 cm HAP column maintained at 65°C and previously equilibrated with 0.035 M-PB. The column was washed with 0.125 M-PB and hybrids together with reassociated DNA were eluted with 0.3 M-PB, pH 6.8, and submitted to a rehybridization procedure. This consisted of hydrolysis of rRNA in 0.2 M-NaOH for 2 hours at 65°C, removal of ribonucleotides by HAP chromatography (0.035 M-PB), elution of single-stranded DNA by 0.125 M-PB, and rehybridization with a fresh portion of [^{3}H]rRNA (50μg/ /ml). The whole procedure was repeated 5 - 6 times. The concentration of DNA was decreased after each step and gradual elimination of sequences non-complementary to rRNA took place.

Hybrids purified by HAP were additionally submitted to chromatography on BD-cellulose. BD-cellulose was obtained according to the procedure of Gillam *et al.* (1967) and Sedat *et al.* (1967, 1969). The column was prepared as described by Udvardy & Venetianer (1971).

DNA-[^{3}H]rRNA hybrids after dialysis against 0.3 M-NaCl were applied onto a 2×12 cm BD-cellulose column equilibrated with 0.3 M-NaCl - 0.02 M-Tris-HCl, pH 7.0. The elution was carried out with a linear gradient of NaCl (0.3 - 1.1 M) and acetone (0 - 18%) in 0.02 M-Tris-HCl buffer, pH 7.0. Hybrids were eluted by 0.54 - 0.6 M-NaCl.

Hybridization of rRNA with DNA reassociated to various C_0t values. Three DNA fractions differring in the degree of repetitivity were obtained as described by Szala & Chorąży (1972). The fast fraction reassociated to $C_0t = 10^{-2}$ was rejected. The intermediate fraction ($C_0t = 10^{-1} - 10^3$) and slow fraction (C_0t above 10^4) were dialysed against 2×SSC, denatured thermally and hybridized in solution with [^{3}H]rRNA in 6×SSC. At proper time intervals 50 μl samples were withdrawn, digested with RNase A and T_1 (as described above), spotted onto 2 cm filters (Whatman no. 1 paper), dried, and left overnight in cold 5% trichloroacetic acid (TCA). Filters were then washed with a fresh portion of 5% TCA, washed twice with 95% ethanol, dried under an infrared lamp and the radioactivity counted in a scintillation counter.

Ultracentrifugation of DNA and DNA-rRNA hybrids in CsCl. To 8 ml of saturated solution of CsCl in 0.01 M-Tris-HCl, pH 8.0, was added 2.5 ml of DNA solution, bringing the final CsCl concentration to 1.70 g/cm^3. The final concentration of DNA was about 0.5 E_{260} unit per 1 ml. The sample was overlaid with 2 ml of paraffin oil, and centrifuged in polyallomer tube in a Beckman rotor no. 30 at 27 000 r.p.m., at 20°C for 96 h. Fractions of 10 drops were collected by siphoning. Corresponding fractions from several tubes were combined, dialysed against 0.1 × SSC, submitted to alkaline denaturation, trapped on millipore filters and hybridized with labelled rRNA.

DNA-rRNA hybrids were isolated in a similar way, but the final CsCl concentration was 1.73 g/cm^3 and the samples were centrifuged in Beckman rotor no. 50 for 48 h at 40 000 r.p.m. and room temperature.

The density of the gradient was estimated by refractometry according to Flamm *et al.* (1972).

Electron microscopy of DNA-rRNA hybrids. For electron microscopic examination, DNA-rRNA hybrids were prepared according to the method of Kleinschmidt & Zahn (1959) modified as follows. Hybrids at a concentration of 5 μg/ml in 1 M-ammonium acetate were mixed with the same volume of 0.02% cytochrome *c* in 1 M-ammonium acetate. This mixture was layered over the surface of 0.3 M-ammonium acetate previously cleaned and covered with talcum. The hybrid-protein film was picked up onto copper grids coated with carbon. Preparations were stained for 30 sec with uranyl acetate (10^{-5} M-uranyl acetate, 6×10^{-5} M-HCl in 100% ethanol) and then shadowed with platinum : palladium (4 : 1) in a vacuum evaporator (VEB Hochvacuum Dresden, Dresden, G.D.R.) at an angle of 6 - 7° during continuous rotation at a speed of 60 turns per minute. The specimens were examined in TESLA BS 513 electron microscope. The microscope was calibrated with latex spherules 1.099 μ in diameter (Serva, Entwicklungslabor, Heidelberg, G.F.R.). The original electronograms were enlarged 5 times, and the length of hybrid molecules were measured.

Radioactivity counting. Liquid samples were counted in a scintillation liquid composed of 3 g PPO and 0.1 g POPOP in 1 litre of toluene - ethylene glycol mixture (6:7, v/v). Radioactivity on filters was measured by placing filters in a liquid composed of 5 g PPO and 0.3 g POPOP in 1 litre of toluene. Radioactivity was counted in a Packard 3380 spectrometer.

RESULTS AND DISCUSSION

The amount of DNA complementary to rRNA. rRNA isolated both from rat liver and rat fibroblasts by a modified Kirby (1965) procedure as described under Materials and Methods revealed on acrylamide gels two distinct homogeneous peaks corresponding to 28S and 18S rRNA (Fig. 1). The average G+C content of rRNA determined by spectrophotometry was 64.3% and from radioactivity of ^{32}P-labelled

rRNA, 65.1%. These values were slightly higher as compared to the value given by Petermann (1964).

By filter hybridization 0.04% of DNA was saturated with [^{3}H]rRNA after a 6 h incubation at a rRNA: DNA ratio of 0.5 (Fig. 2) which was shown to be sufficient to saturate all complementary rRNA sequences. In further experiments, the incubation time was 16 h. Assuming a complexity for the rat haploid genome

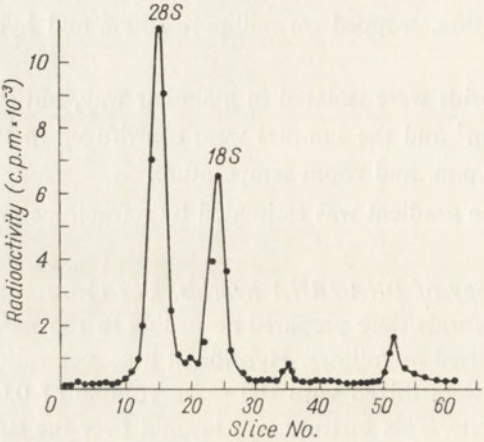


Fig. 1. Polyacrylamide-gel electrophoresis of [^{32}P]rRNA isolated from rat embryo fibroblasts.

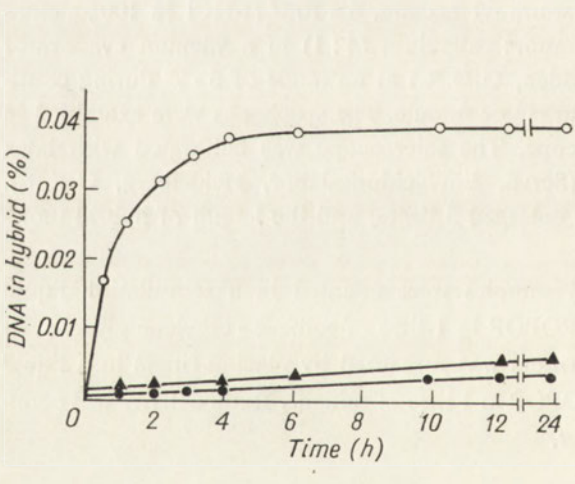


Fig. 2

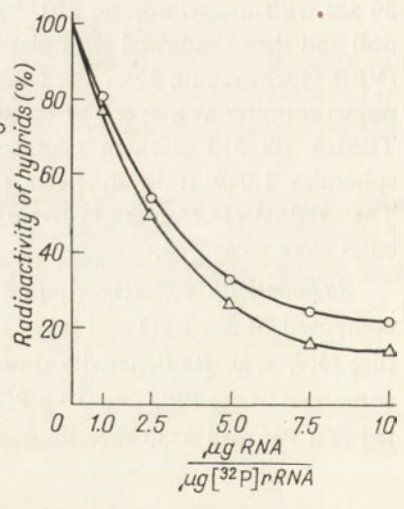


Fig. 3

Fig. 2. Hybridization of [^{3}H]rRNA with DNA fixed on Millipore filter at the DNA to rRNA ratio of 0.5. rRNA was incubated with filter loaded with rat liver DNA (○), *E. coli* DNA (▲) or with filter alone (●). The results obtained with rat liver DNA were corrected by subtracting the counts bound to blank filters or filters loaded with *E. coli* DNA.

Fig. 3. Competition curves of unlabelled rRNA (mixture of 28S and 18S rRNA at 1:1 weight ratio) with [^{32}P]rRNA. Hybridization was done on Millipore filters. The two curves are from two independent experiments and each point represents an average from two determinations.

of 1.8×10^{12} daltons, and a combined molecular weight of the two rRNA subunits to be 2.23×10^6 daltons (Petermann & Pavlovec, 1966), 0.04% of hybridized DNA would represent 300 copies of rRNA genes. This estimate is in agreement with previously found values (Steele, 1968; Mohan *et al.*, 1969), but exceeds by about 1.7 times the values reported by Brimacombe & Kirby (1968) and Melli *et al.* (1971). Following the addition of unlabelled rat liver rRNA to the hybridization mixture a distinct competition effect was noted (Fig. 3) indicating the specificity of hybridization reaction.

Hybridization of rRNA to the DNA fraction obtained by CsCl density gradient centrifugation. Rat liver DNA submitted to CsCl equilibrium centrifugation was found as a symmetrical band at 1.698 g/cm³. The hybridization of the DNA fractions obtained along the CsCl gradient with [³H]rRNA revealed that complementary sequences are located at the heavy side of the main band, in the region of 1.709 g/cm³ (Fig. 4). This is in good agreement with the value of 1.708 g/cm³ found by Steele (1968) in the rat for nucleolar DNA fractions complementary to rRNA. No hybrid formation was noted with the main band (Fig. 4). This means that sequences complementary to rRNA are rather clustered and not dispersed through the genome.

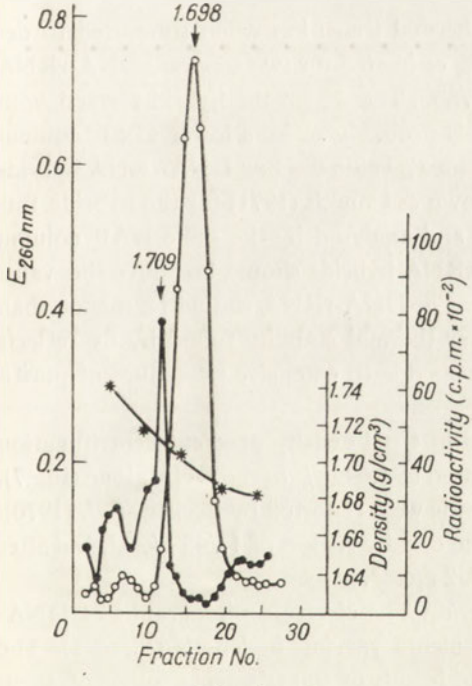


Fig. 4

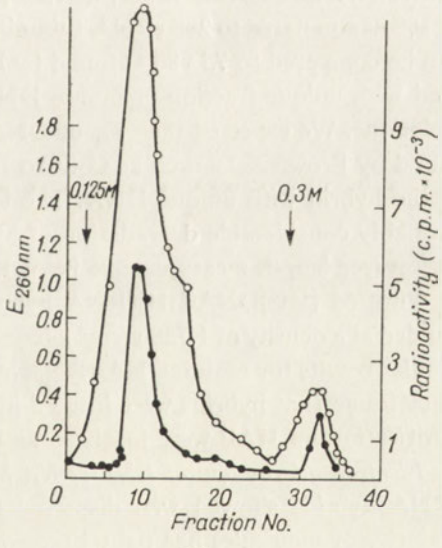


Fig. 5

Fig. 4. Hybridization of [³H]rRNA with DNA fractions obtained by CsCl gradient equilibrium centrifugation. $E_{260\ nm}$ (○); radioactivity of rRNA-DNA hybrids (●); density of CsCl (*).

Fig. 5. Typical elution profile from HAP column of DNA-[³H]rRNA hybrids. The first peak, eluted with 0.125 M-PB, contained products formed by endonuclease S_1 and RNase A and T_1 digestion, the second peak eluted with 0.3 M-PB, contained DNA-rRNA hybrids and reassociated DNA. $E_{260\ nm}$ (○); radioactivity (●).

Isolation and features of DNA sequences complementary to rRNA. Five to six cycles of hybridization and chromatography of DNA-rRNA hybrids on HAP column as described in Materials and Methods led to concentration of sequences complementary to rRNA. These sequences submitted to additional purification on BD-cellulose showed a considerable degree of purity as judged by the following criteria. The amount of DNA eluted with 0.3 M-PB from HAP column (Fig. 5) dropped at each step of rehybridization in such a way that DNA C_0t calculated for the successive steps decreased steadily forming the series: 20, 6.4, 3.2, 1.3, 0.64, 0.19. This means that the contribution of reassociated double-stranded DNA to the material tightly bound to HAP column and eluted with 0.3 M-PB decreased at each hybridization step while the addition of rRNA (50 μg/ml) at each step warranted that the reaction was of the "RNA-driven" type. Using [^{14}C]DNA and [^{3}H]rRNA it was calculated that the DNA to rRNA weight ratio of the "final" hybridization product, i. e. after 5 hybridization steps each followed by HAP chromatography, and 2 cycles of purification on BD-cellulose, was 1.1:1. This means that the hybrid preparation was still contaminated with about 10% of double-stranded DNA. This figure may be underestimated as the DNA preparation used in this experiment was labelled in thymidine.

Hybrid preparations showed a sharp thermal transition when submitted to denaturation on HAP column (Fig. 6). The T_m estimated for two separate DNA-rRNA hybrid preparations was in the range 75 - 78°C. The T_m of the hybrid formed with 18S rRNA was slightly lower, at 75°C; this probably reflects lower G+C content of 18S as compared to 28S rRNA subunit. The T_m value for our DNA-rRNA hybrids can be compared to 75 - 80°C found by Brown & Church (1971) for the hybrids formed with unique fraction of mouse DNA and analysed by the same HAP column technique. We expected that T_m of DNA-rRNA hybrids should be above the value found by Brown & Church as G+C content in DNA-rRNA should be higher than in the hybrid with unique DNA. The lower thermal stability found by us reflects probably considerable degradation of hybrids due to extensive procedure of purification (see length measurements below).

Purified [^{14}C]DNA-[^{3}H]rRNA hybrids in CsCl density gradient centrifugation banded at a density of 1.728 g/cm^3 as compared to 1.699 g/^{3} of the DNA alone (Fig.7). Similar results for mouse DNA-rRNA hybrids were reported by Becker *et al.* (1970). They found that hybrids were located in the density region of 1.745 g/cm^3, but after purification on HAP were localized at 1.732 g/cm^3.

Electron microscopy of DNA-rRNA hybrids. The electron micrographs of DNA-rRNA hybrids showed well dispersed filaments varying in length (Plate 1). The majority of molecules has paired the whole length of the filaments, some of them having short segments sticking out. These segments probably represented undigested fragments of DNA or RNA. The length of 236 hybrid molecules was measured. The histogram of length distribution showed that the length of the majority of molecules fell in the regions from 0.2 μm to 0.5 μm (Fig. 8). Only a few molecules with the length above 1 μm were found; they probably represent unbroken ribosomal RNA molecules hybridized with homologous DNA regions. Assuming that the

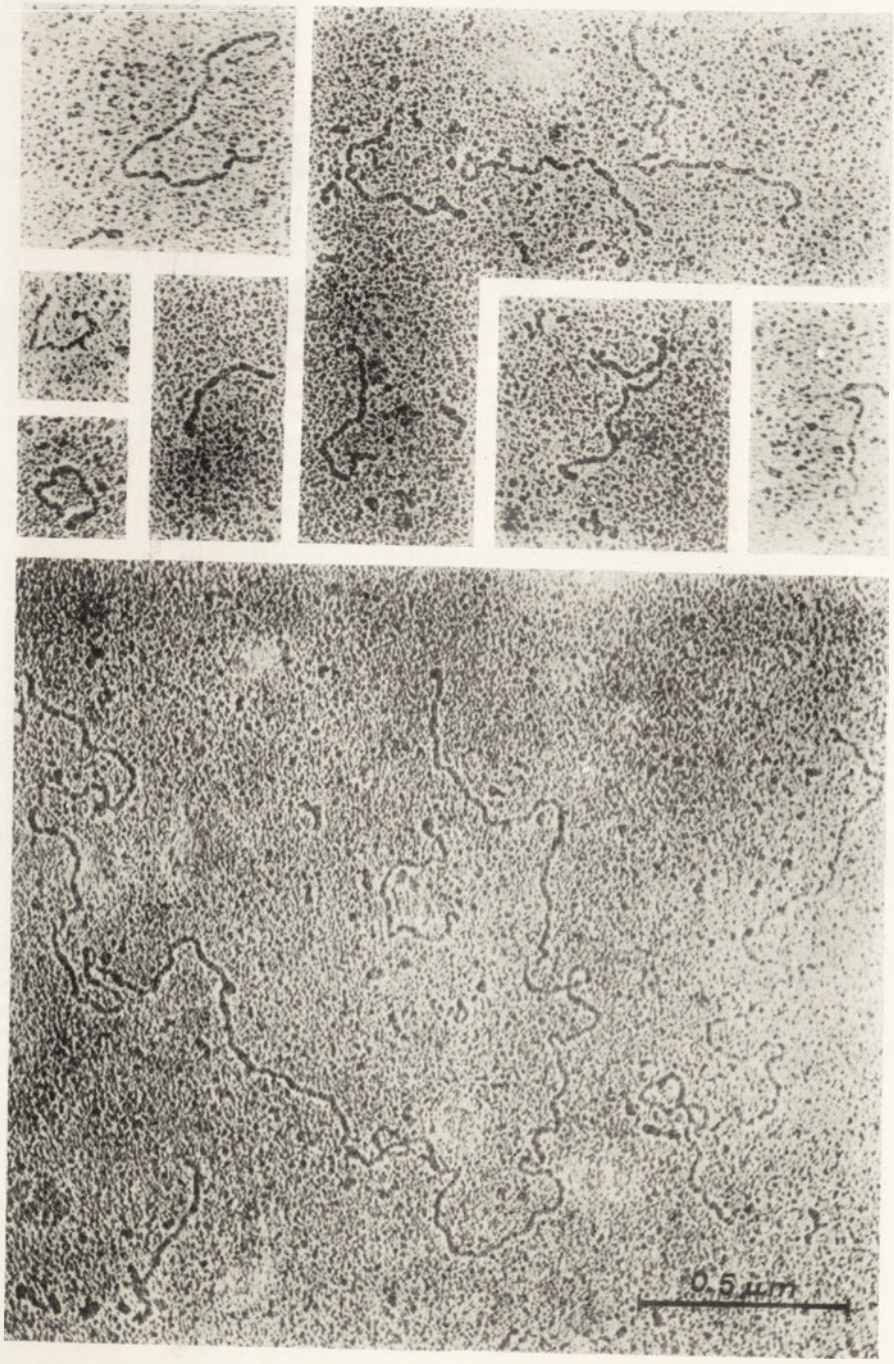


Plate 1. Electron micrographs of DNA-rRNA hybrids showing molecules of various length. The bar indicates 0.5 µm.

M. Hanausek-Wałaszek *et al.* (*facing p. 52*).

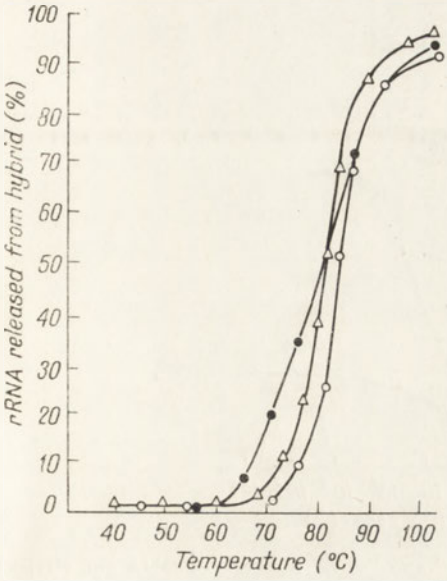


Fig. 6

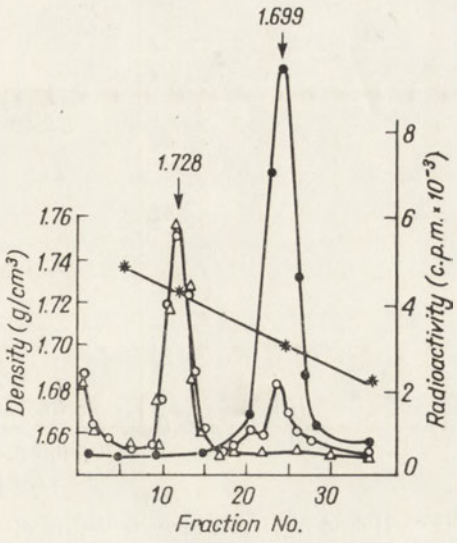


Fig. 7

Fig. 6. Melting profiles of DNA-[^{3}H]rRNA (△), DNA-[^{32}P]rRNA (○) hybrids, and DNA-18S[^{3}H]rRNA (●), adsorbed on HAP column.

Fig 7. CsCl density gradient centrifugation of DNA and DNA-rRNA hybrids. [^{14}C]DNA alone (●); (△) and (○) two independent samples of [^{14}C]DNA-[^{3}H]rRNA hybrids. CsCl density (∗).

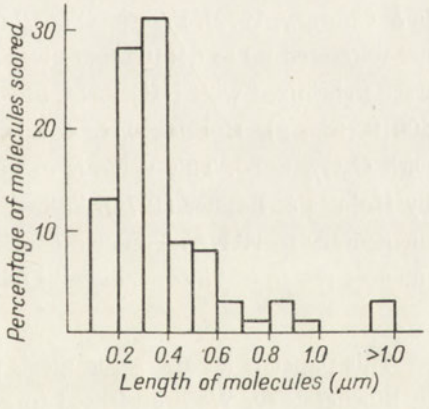


Fig. 8. Histogram of length distribution of 236 hybrid molecules.

base-to-base distance of the hybrid amounts to 2.96 Å (Robberson *et al.*, 1972; Murphy & Attardi, 1974), the linear density of the hybrid molecule will be 220 daltons per 1 Å of length. Thus our hybrid molecules have a size ranging from 4×10^5 to 1×10^6 daltons. This corresponds to 500 - 1600 nucleotide pairs. It is obvious that noticeable degradation of DNA sequences complementary to rRNA took place during the multi-step purification procedure. Similar values for DNA-rRNA hybrid size (2.2×10^5 - 3.5×10^6 daltons) were found by Spadari *et al.* (1972) in sedimentation analysis.

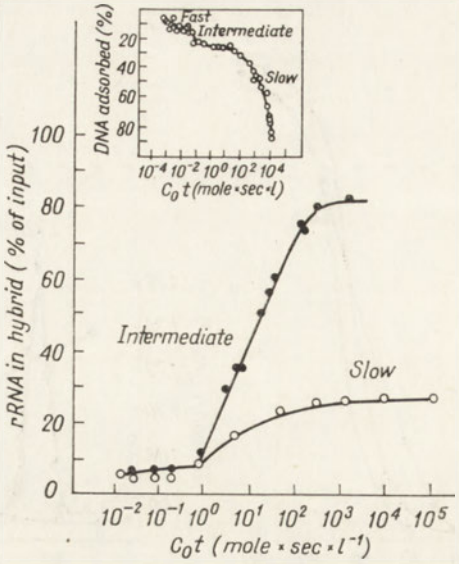


Fig. 9. Hybrid formation between "intermediate" and "slow" fractions of DNA and [^{3}H]rRNA in DNA excess. Hybridization with "intermediate" fraction (●) was carried out in 2×SSC and with "slow" fraction (○) in 6×SSC.
Inset shows the reassociation curve of rat DNA (from: Szala & Chorąży, 1972).

Hybridization of rRNA with DNA fractions. Rat DNA was fractionated into three arbitrary fractions using HAP chromatography as described elsewhere (Szala & Chorąży, 1972). Fraction "fast" (see inset in Fig. 9) containing sequences which are reiterated up to 6×10^6 times was rejected. Fractions "intermediate" and "slow" were hybridized with [^{3}H]rRNA in solution at the DNA to rRNA weight ratio of 500:1 (Fig. 9). Hybrids were formed preferencially with "intermediate" fraction with $C_0t_{1/2} = 50$. This value is in good agreement with $C_0t_{1/2} = 37$ as estimated by Holmes & Bonner (1974). "Slow" fraction contained much less sequences complementary to rRNA. Thus we conclude that the major part of coding rRNA sequences reside in "intermediate" DNA.

This paper is a part of the thesis prepared by M.H.W. for the degree of Ph.D. in Biochemistry. We are pleased to acknowledge the skilful technical assistance of Mrs. Barbara Sikorska and useful comments of Dr. S. Szala. We are greatly indebted to Dr. Susan Gerbi for reading the manuscript and remarks.

REFERENCES

Becker, W.M., Hell, A., Paul, J. & Williamson, R. (1970) *Biochim. Biophys. Acta*, **199**, 348 - 362.
Birnstiel, M.L., Wallace, H., Sirlin, J.L. & Fischberg, M. (1966) *Natl. Cancer Inst. Monogr.*, **23**, 431 - 447.

Birnstiel, M.L., Speirs, J., Purdom, I., Jones, K. & Loening, U. (1968) *Nature* (*London*), **219**, 454 - 463.
Brimacombe, R.L.C. & Kirby, K.S. (1968) *Biochim. Biophys. Acta*, **157**, 362 - 367.
Brown, I.R. & Church, R.B. (1971) *Biochem. Biophys. Res. Commun.*, **42**, 850 - 875.
Brown, D.D. & Blacker, A. (1972) *J. Mol. Biol.*, **63**, 75 - 85.
Brown, D.D. & Weber, C.S. (1968a) *J. Mol. Biol.*, **34**, 661 - 680.
Brown, D.D. & Weber, C.S. (1968b) *J. Mol. Biol.*, **34**, 681 - 697.
Dawid, I.B., Brown, D.D. & Reeder, R.H. (1970) *J. Mol. Biol.*, **51**, 341 - 360.
Flamm, W.G., Birnstiel, M.L. & Walker, P.M.B. (1972) in *Subcellular Components Preparation and Fractionation* (G.D. Birnie, ed.) pp. 279 - 310. Butterworths, London; University Park Press, Baltimore.
Gall, J.E. (1974) *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3078 - 3081.
Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E. & Tener, G. (1967) *Biochemistry*, **6**, 3043 - 3056.
Gillespie, D. & Spiegelman, S. (1965) *J. Mol. Biol.*, **12**, 829 - 842.
Holmes, D.S. & Bonner, J. (1974) *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1108 - 1115.
Katz, S. & Comb, D.G. (1963) *J. Biol. Chem.*, **238**, 3065 - 3067.
Kirby, K.S. (1965) *Biochem. J.*, **96**, 266 - 269.
Kleinschmidt, A & Zahn, R.K. (1959) *Z. Naturforsch.*, **14b**, 770 - 779.
Loening, U.E. (1967) *Biochem. J.*, **102**, 251 - 257.
Melli, M., Whitfield, C., Roa, K., Richardson, M., & Bishop, J.O. (1971) *Nature* (*London*), **231**, 8 - 12.
Miller, O.L., Jr. & Beatty, B.R. (1969a) *Science*, **164**, 955 - 957.
Miller, O.L., Jr. & Beatty, B.R. (1969b) *J. Cell. Physiol.*, **74**, 225 - 232.
Miyazawa, Y. & Thomas, C.A., Jr. (1965) *J. Mol. Biol.*, **11**, 223 - 237.
Mohan, J., Dunn, A. & Casola, L. (1969) *Nature* (*London*), **223**, 295 - 296
Murphy, W.J. & Attardi, G. (1974) *J. Mol. Biol.*, **90**, 65 - 76.
Nygaard, A.P. & Hall, B.D. (1964) *J. Mol. Biol.*, **9**, 125 - 142.
Palacios, R., Palmiter, R.D. & Schimke, R.T. (1972) *J. Biol. Chem.*, **247**, 2316 - 2321.
Petermann, M.L. (1964) *The physical and chemical properties of ribosomes*, pp. 103 - 108. Elsevier Pub. Co, Amsterdam, London, New York.
Petermann, M.L. & Pavlovec, A. (1966) *Biochim. Biophys. Acta*, **114**, 264 - 276.
Quagliarotti, G. & Ritossa, F. (1968) *J. Mol. Biol.*, **36**, 57 - 69.
Reeder, R.H. & Brown, D.D. (1970) *J. Mol. Biol.*, **51**, 361 - 377.
Ritossa, F.M. & Spiegelman, S. (1965) *Proc. Natl. Acad. Sci. U.S.A.*, **53**, 737 - 745.
Robberson, D., Aloni, Y., Attardi, G. & Davidson, N. (1972) *J. Mol. Biol.*, **64**, 313 - 317.
Savitsky, J.P. & Stand, F. (1966) *Biochim. Biophys. Acta*, **114**, 419 - 422.
Scheer, U., Trendelenburg, M.T. & Franke, W.W (1975) *J. Cell Biol.*, **65**, 163 - 179.
Sedat, J.W., Kelly, R.B. & Sinsheimer, R.L. (1967) *J. Mol. Biol.*, **26**, 537 - 540.
Sedat, J.W., Lyon, A. & Sinsheimer, R.L. (1969) *J. Mol. Biol.*, **44**, 415 - 434.
Spadari, S., Mazza, G. & Falaschi, A. (1972) *Eur. J. Biochem.*, **28**, 389 - 398.
Steele, W.J. (1968) *J. Biol. Chem.*, **243**, 3333 - 3341.
Sutton, W.D. (1971) *Biochim. Biophys. Acta*, **240**, 522 - 531.
Szala, S. & Chorąży, M. (1972) *Acta Biochim. Polon.*, **19**, 235 - 250.
Szala, S., Chorąży, M. & Kilarski, W. (1971) *FEBS Lett.*, **17**, 96 - 99.
Tartoff, K.D. & Perry, R.P. (1970) *J. Mol. Biol.*, **51**, 171 - 183.
Udvardy, A. & Venetianer, P. (1971) *Eur. J. Biochem.*, **20**, 513 - 517.
Wellauer, P.K. & Dawid, I.B. (1974) *J. Mol. Biol.*, **89**, 379 - 395.
Wellauer, P.K., Reeder, R.H., Carrol, D., Brown, D.D. & Deuth, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2823 - 2827.
Yao, M.C., Kimmel, A.R. & Gorovsky, M.A. (1974) *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3082 - 3086.

NIEKTÓRE CECHY HYBRYDÓW DNA-rRNA SZCZURA

Streszczenie

1. W DNA z wątroby szczura sekwencje komplementarne do rRNA wynoszą 0.04%, co stanowi około 300 genów rRNA na genom haploidalny.

2. Stosunek wagowy DNA:rRNA oczyszczonych hybrydów DNA-rRNA jest 1,1:1. Temperatura topnienia hybrydów oznaczona na kolumnie hydroksyapatytu wynosi 75 - 80°C. W gradiencie CsCl hybrydy plasowały się w gęstości 1.728 g/cm^3, podczas gdy główna masa DNA w gęstości 1.699 g/cm^3.

3. Histogram rozkładu długości cząsteczek hybrydu DNA-rRNA sporządzony na podstawie obrazów w mikroskopie elektronowym wykazał, że większość cząsteczek miała długość od 0.2 do 0.5 μm, co odpowiada 4×10^5 - 1×10^6 daltonów, tj. 670 - 1600 par nukleotydów.

4. Sekwencje DNA komplementarne do rRNA są zlokalizowane po stronie cięższej głównej masy DNA i plasują się w gęstości CsCl = 1.709 g/cm^3. Sekwencje te są zawarte we frakcji DNA reasocjującej w $C_0t = 10^{-1}$ - 10^2.

Received 10 October, 1975.

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 1

CELINA JANION

THE SYNTHESIS AND PROPERTIES OF N^6-SUBSTITUTED 2-AMINOPURINE DERIVATIVES*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36; 02-532 Warszawa, Poland

1. The synthesis, ultraviolet absorption spectra, and behaviour in alkali of N^6-methoxy-, N^6-methyl,hydroxy-, and N^6-hydroxy-2-aminopurines have been described.

2. N^6-Methoxy-2-aminopurine riboside 5′-pyrophosphate has been prepared and used for polymerization with polynucleotide phosphorylase.

3. The copolymer containing N^6-methoxy-2-aminopurine riboside and adenosine residues has been obtained; attempts to synthesize the homopolymer have not been successful.

4. All the purine analogues synthesized have been tested and shown to act mutagenically on *Salmonella typhimurium* TA1530.

The unique role and importance of complementary bases and hydrogen bonding in the processes of replication, recombination, mutation, transcription, and translation of nucleic acids, led to the synthesis of different kinds of modified bases, nucleosides, and polynucleotides. Many of these compounds interfere with natural nucleic acid derivatives and act as antimetabolites.

The most varied types of hydroxypurine derivatives were synthesized by Giner-Sorolla *et al.* (1966, 1968, 1972). Some of these compounds, e.g. N^6-hydroxypurine, N^6-methoxypurine, or N^6-hydroxy-2-aminopurine, exert a strong antimetabolic and growth inhibitory effect on tumours (Sartorelli *et al.*, 1963, 1964; Giner-Sorolla *et al.*, 1968, 1972). Some of them have proven mutagenic for T_4 phage (Freese, 1968).

In this paper the synthesis and physico-chemical properties of N^6-methoxy-, N^6-methyl,hydroxy-, and N^6-hydroxy-2-aminopurine derivatives[1] are described, and their potential mutagenic activity has been tested. The synthesis of N^6-methoxy-2-aminopurine riboside 5′-pyrophosphate and attempts to polymerize it by polynucleotide phosphorylase are also described.

* This investigation was supported by the Polish Academy of Sciences within the project 09.3.1, and in part by the Agricultural Research Service, U.S. Department of Agriculture (FG-Po-307).

[1] Abbreviations used are: N^{6}OMe2AP, N^6-methoxy-2-aminopurine; N^{6}MeOH2AP, N^6-methyl,hydroxy-2-aminopurine; N^{6}OH2AP, N^6-hydroxy-2-aminopurine; 6C12AP, 6-chloro-2-aminopurine; OMeHA, *O*-methylhydroxylamine; NMeHA, *N*-methylhydroxylamine; HA, hydroxylamine.

EXPERIMENTAL

Chemicals. 6-Chloro-2-aminopurine and its riboside were purchased from Waldhof (Mannheim, G.F.R.); *N*-methyl-, *O*-methyl, and hydroxylamine (all compounds as HCl salts), from Eastman Kodak Co. (Rochester, N.Y., U.S.A.).

Enzymes. Polynucleotide phosphorylase of *E. coli* (Williams & Grunberg-Manago, 1964) was obtained by courtesy of Dr. Grunberg-Manago, that of *M. luteus* was purchased from Boehringer (Mannheim, G.F.R.), and calf spleen nucleoside phosphorylase from Sigma Chemicals Co. (St. Louis, Mo., U.S.A.).

Chromatography. Excellent resolution of the products from starting material was obtained on Eastman-Kodak t.l.c. cellulose sheets dipped in the mixture of H_2O - sat. $(NH_4)_2SO_4$ (9:1, v/v) which after drying were developed in the mixture of ethanol - H_2O (8:2, v/v; Lane, 1963). The same procedure and solvent with ascending paper chromatography (Whatman no. 1) gave the following R_F values: N^6OMe2AP (I), 0.4; its riboside, 0.62; N^6MeOH2AP (II), 0.28; N^6OH2AP (III), 0.11; 6C12AP, 0.72. With ethanol - 1 M-ammonium acetate, R_F values were: I, 0.82; riboside of I, 0.73; 6-thiomethyl-2-aminopurine riboside, 0.73; II, 0.80; III, 0.52; 6C12AP, 0.89.

Colour test. For characterization of compounds on chromatograms in addition to the use of u.v. lamp, two spray reagents were used: (a) Ehrlich reagent (1 g of dimethylaminobenzaldehyde + 10 ml of conc. HCl + ethanol to a final volume of 100 ml), and (b) modified for chromatography spray, Pauly reagent (diazotized sulphanilic acid) followed by 5% of sodium carbonate (Tabor, 1971). With the Ehrlich reagent the synthesized compounds I, II, and III, as well as guanine and adenine, give yellow spots; Pauly reagent gave yellow spots with adenine and III, with guanine an orange spot, with I a pink spot, and no colour with II.

Syntheses. N^6OMe2AP (I): 50 mg of 6C12AP in 8 ml of 3 M-OMeHA, adjusted to pH 4.5, were kept in a boiling-water bath for about 2 h. During the course of the reaction 6C12AP progressively dissolves. Cooling gave crystals of I. These were washed, and recrystallized twice from water. The m.p. was 218°C. Found: C, 30.7; H, 4.72; N, 35.81. Calculated for $N_6C_6ClH_9O,H_2O$: C, 30.41; H, 4.8; N, 35.5%.

N^6MeOH2AP (II): 6C12AP when boiled with NMeHA almost immediately led to formation of compound II. 6C12AP, 50 mg, in 8 ml of 2.5 M-NMeHA, pH 4.5, were warmed in a boiling-water bath for about 20 min, or kept at 60°C for 60 min. The appearing fluffy material was filtered off, the solution concentrated, and the product crystallized twice from cold ethanol - acetone. Warming II in ethanol led to destruction of the product and disappearance of u.v. absorption. Found: C, 32.22; H, 4.69; N, 25.57. Calculated for: $N_6C_6Cl_2H_{10}O,H_2O$: C, 31.0; H, 4.46; N, 26.57%.

N^6OH2AP (III): 50 mg of 6C12AP in 8 ml of 3 M-HA, pH 4.5, were warmed in a boiling-water bath for about 2 h. When necessary, the solution was clarified by filtration and the product was crystallized twice from water.

Riboside of N^6OMe2AP: 50 mg of 6C12AP-riboside (0.17 mmole) was dissolved in 4 ml of 2.5 M-OMeHA (10 mmoles), pH 4.5, and kept at 37°C for about 24 h;

the solution was then neutralized with NH_4OH, concentrated, passed through a Sephadex G-10 column and eluted with water. Riboside of compound I was collected, evaporated and submitted to phosphorylation. The attempts to crystallize riboside of compound I were unsuccessful. The product readily darkened on exposure to light.

5'-Phosphate riboside of N^6OMe2AP: This was prepared by enzymatic phosphorylation of ribonucleoside with wheat shoots phosphotransferase (Barner & Cohen, 1959). Pilot experiments showed that 6C12AP-riboside is a much better substrate than N^6OMe2AP-riboside (60% *versus* 30% phosphorylation rate). Thus 6C12AP-riboside was phosphorylated first and then reacted with OMeHA. It should be noted that the optimal reaction time at 37°C for the riboside of 6C12AP was 2 - 3 hours, and for the riboside of N^6OMe2AP, 1 hour. After that time both products underwent rapid dephosphorylation. This was not observed with other substrates like 2'-*O*-methylcytidine (Janion *et al.*, 1970), or the riboside of 2-aminopurine (Janion & Shugar, 1973), where prolongation of the reaction time up to 24 hours did not appreciably influence the reaction yields.

The reaction mixture contained: 6C12AP-riboside, 100 mg; *p*-nitrophenol, 1.7 g; wheat shoots water extract, 25 ml; 0.2 M-phosphate buffer, pH 4.0, 15 ml. It was found necessary to add some conc. HCl (~15 µl) to bring the whole of the mixture to pH 4.0. The resulting 5'-nucleotide was then separated by chromatography on Whatman no. 17 paper (10 - 12 sheets) in ethanol – 1 M-ammonium acetate (7:3, v/v) solvent, eluted, concentrated and reacted with OMeHA. The 5'-phosphoriboside of N^6OMe2AP formed was freed from excess OMeHA by passage through a Sephadex G-10 column, and further purified on Dowex H^+ (50 W × 8, 100 - 200 mesh). The last step after prolonged extraction with water gave the free acid of N^6OMe2AP-ribotide in excellent purity, highly suitable for pyrophosphorylation.

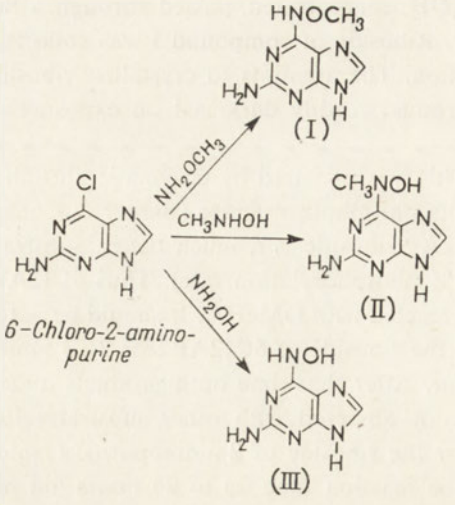
RESULTS

The syntheses of N^6-substituted 2-aminopurine derivatives were accomplished by reaction of 6-chloro-2-aminopurine compounds with OMeHA, NMeHA, or HA in aqueous solution (Scheme 1). The replacement of 6-chloro on HA derivatives occurs in a broad range of pH (3.5 - 7.5); the reaction time depends on the pH used.

More detailed studies on the dependence of the reaction time on pH, were conducted with 6C12AP-riboside and 2.5 M-OMeHA, at 37°C. The reaction over the pH range 3.5 - 5.0 was completed after about 24 h; at pH 6.5 - 7.0 after 72 h. Therefore pH 4.5 was selected for the preparation of all synthesized compounds.

The reaction can be followed by measurement of u.v. absorption spectra, t.l.c. or paper chromatography. Figure 1 shows the difference in u.v. absorption spectra between 6C12AP-riboside and the formed N^6OMe2AP-riboside. The same product was obtained when 6-thiomethyl-2AP-riboside reacted with OMeHA at 37°C, whereas 6-thio-2AP-riboside was inert even when the reaction was conducted at 95°C.

Because of the difficulty in crystallizing N^6OMe2AP-riboside, the reaction product was identified by comparing the chemically obtained N^6OMe2AP with the



I, N^6-methoxy-2-aminopurine (N^6OMe2AP)
II, N^6-methyl,hydroxy-2-aminopurine (N^6MeOH2AP)
III, N^6-hydroxy-2-aminopurine (N^6OH2AP)

Scheme 1

base liberated from N^6OMe2AP-riboside. As it has been found, the riboside undergoes phosphorolysis by purine phosphorylase; the resulting base exhibited chromatographic behaviour and u.v. absorption identical with those of the chemically prepared crystalline N^6OMe2AP (for details see Experimental).

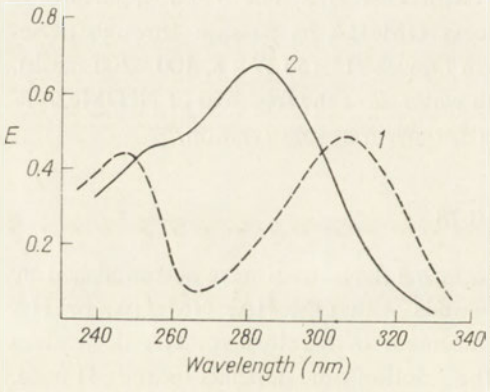


Fig. 1. The reaction between 6-chloro-2-aminopurine riboside and 2.5 M-OMeHA at pH 4.5 and 37°C. Samples (5 µl) of the reaction mixture were diluted to 1 ml with H_2O and the spectra determined: (1), at zero time, (2), after 20 hours.

Ultraviolet spectra. Examination of the u.v. aborption spectra of the obtained purine analogues over a broad range of pH showed that these bases can, like guanine, occur in five different forms. All spectra are shown together in Figures 2 - 4. The spectra of N^6OMe2AP - riboside do not differ from the spectra of its 5′-phosphate and are not included. N^6OMe2AP at pH below zero exists in the dicationic form (Fig. 2a); pK 4.7 is the value for transition of the monocationic to the neutral form (Fig.3a), pK 10.4 and <13 are values for transition of the neutral to the monoanionic (Fig. 4a) and the dianionic form, respectively.

The u.v.-spectral data and some ionization constants are given in Tables 1 and 2, respectively.

Figure 2

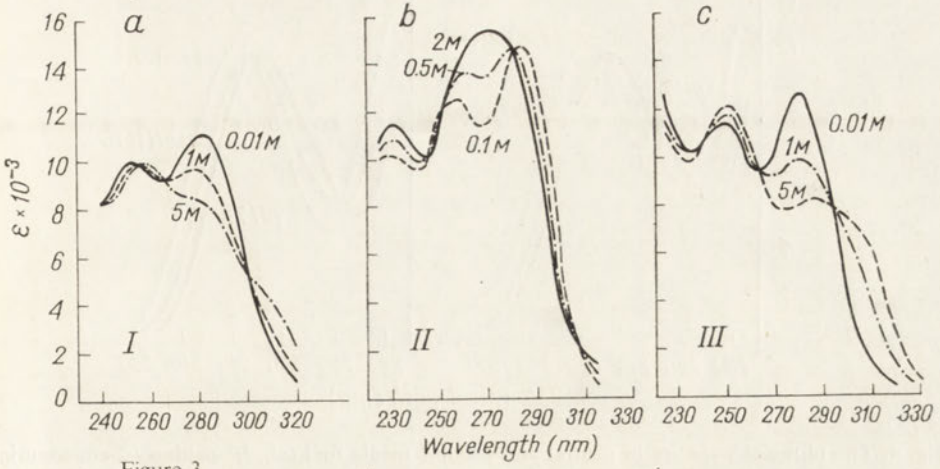


Figure 3

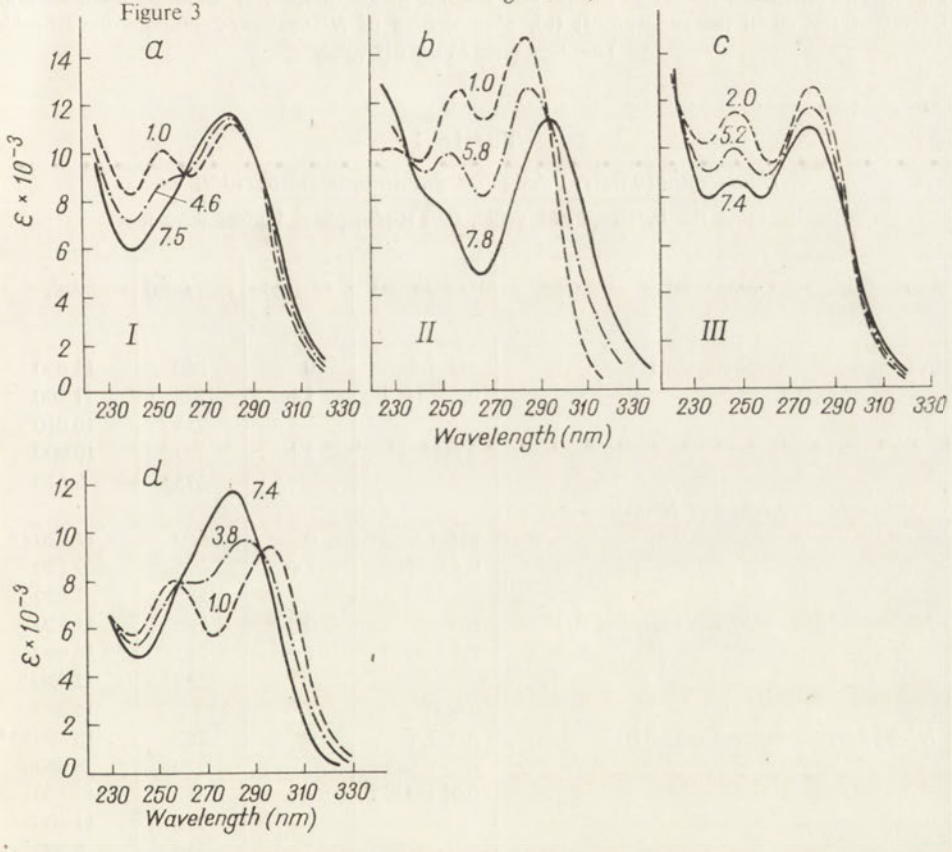


Fig. 2. The ultraviolet spectra of monocationic, dicationic, and intermediate forms for (*a*), N^6-methoxy-2-aminopurine; (*b*), N^6-methyl,hydroxy-2-aminopurine; (*c*) N^6-hydroxy-2-aminopurine, at indicated molarity of HCl.

Fig. 3. The ultraviolet spectra of neutral and monocationic forms, and the pK_1 values for (*a*), N^6-methoxy-2-aminopurine; (*b*), N^6-methyl,hydroxy-2-aminopurine; (*c*), N^6-hydroxy-2-aminopurine; (*d*) 5′-phosphate of N^6-methoxy-2-aminopurine riboside. Numbers refer to the pH value.

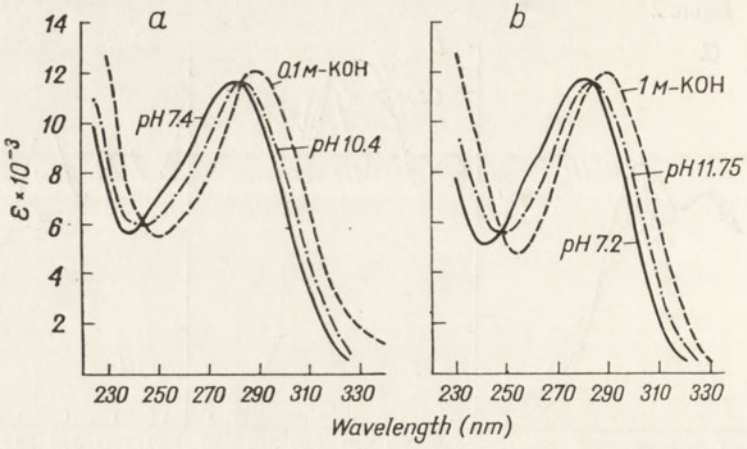


Fig. 4. The ultraviolet spectra in neutral and alkaline media for: (*a*), N^6-methoxy-2-aminopurine (corrected for alkali decomposition); (b), 5′-phosphate of N^6-methoxy-2-aminopurine riboside. Numbers refer to the pH value.

Table 1

Spectrophotometric data for 2-aminopurine derivatives

For maintaining the indicated pH value, 0.01 M-phosphate buffers were used.

Compound	Conditions	Charge	ε_{max} (nm)	λ_{max}
N^6-Methoxy-2-aminopurine (I)	pH 7.5	0	280	11 650
	0.1 M-HCl	1+	282	11 200
			253	10 010
	5 M-HCl	2+	257	10 000
			275*	8 570
5′-Phosphate riboside of N^6-methoxy-2--aminopurine	pH 7.5	0	280	11 770**
	0.1 M-HCl	1+	296	9 320
			225	7 820
N^6-Methyl,hydroxy-2-aminopurine (II)	pH 7.8	0	295	11 320
	0.1 M-HCl	1+	285	14 680
			258	12 530
	2 M-HCl	2+	270-272	15 260
N^6-Hydroxy-2-aminopurine (III)	pH 7.4	0	282	10 880***
			250	8 650
	0.01 M-HCl	1+	282	12 520
			250	11 500
	5 M-HCl	2+	286	8 200
			250	12 070

* Inflection.

** Calculated on the basis of phosphate estimation and compared to 2′(3′)-uridylic acid as a control.

*** λ_{max} for mono and dicationic forms were calculated on the basis of the ε_{max} at 280 nm for neutral form as reported by Giner-Sorolla *et al.* (1966).

Table 2

The pK values of 2-aminopurine derivatives

pK_1 and pK_2 values are for transition of neutral species to cationic and anionic form, respectively.

Compound	pK_1	pK_2
N^6OMe2AP (I)	4.6	10.4
5′-Phosphate riboside of I	3.8	11.9
N^6MeOH2AP (II)	5.8	
N^6OH2AP (III)	5.2	

No special attempts were made to estimate the tautomeric forms and position of protonation in the cationic species. All compounds in the monocationic form show similar spectra so that it is possible that in all cases the first proton is localized at the same site in each molecule. However, whereas the dicationic forms of I and III are similar, they differ distinctly from the dicationic form of II (Fig. 2). This must reflect some difference at least in the second proton localization site. As II exists in the fixed N^6-amino form, another tautomeric state for I and III, and hence differences in acceptance site of second proton, should be considered.

N^6OMe2AP possesses two protons which can dissociate and make a contribution to formation of anionic species, viz. $N_{(1)}$ (or N^6) in the pyrimidine part, and $N_{(7)}$ or $N_{(9)}$ in the imidazole part. Comparison of the monoanion spectrum of N^6OMe2AP with that for 5′-phosphate riboside derivative, where only one proton from the pyrimidine part can dissociate, suggests that, as for guanine, the pyrimidine proton of N^6OMe2AP dissociates first (compare Fig. 4a and b). Transition of the monoanionic to the dianionic form only slightly affects the u.v. spectrum of N^6OMe2AP, but appreciably increases its absorption at λ_{max} (not shown).

The alkali instability of the tested purine bases renders difficult a more detailed investigation of their anionic species.

Stability. All the investigated bases are quite stable in acid medium. There were no changes in u.v. spectra after storing I, II or III in 5 M-HCl at room temperature. By contrast, the free bases were extremely alkali-labile.

For N^6OMe2AP the alkali transition begins at a pH of about 10 and is greatly accelerated by the presence of oxygen. The dependence of the alkali transition on oxygen is more evident when a concentrated solution of a base is used.

Figure 5 shows the changes in the u.v. absorption spectrum of N^6OMe2AP (I) in 1 M-KOH at room temperature. The transition is complete within about 3 hours, following which the u.v. spectrum remains constant. Simultaneously the colourless solution darkens.

The well-defined three isosbestic points lead one to conclude that in 1 M-KOH only one compound is formed. But attempts to isolate it were unsuccessful. During chromatography even in alkaline solvent [isopropanol - H_2O - NH_3 (or 1 M-KOH), 7:2:1] several spots, some coloured, some blue or fluorescent visible under u.v. lamp, are formed. Most of these spots after elution and acidification release bubbles and

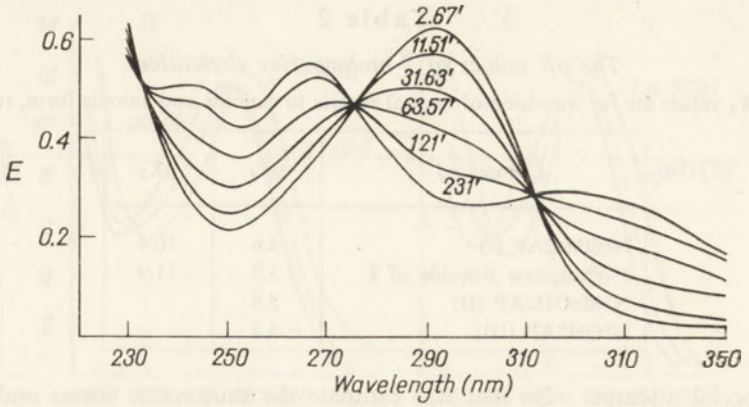


Fig. 5. The alkali transition of N^6-methoxy-2-aminopurine in 1 M-KOH. The u.v. spectra were measured on a Cary 118 instrument, and the reaction followed by the decrease of extinction at 290 nm with time.

ammonia was detected. Obviously the compound formed in 1 M-KOH is not stable in the less alkaline medium and undergoes further degradation.

The riboside or 5′-phosphate riboside of I is not affected even by 1 M-KOH.

N^6MeOH2AP (II) and N^6OH2AP (III) show similar alkali sensitivities as compound I. The changes in the u.v. absorption spectrum of II in 1 M-KOH indicate that more than one compound is formed. The colour of the solution changes to a characteristic deep green, altering to bright-red in transmitted light.

Unexpectedly, compound III in 1 M-KOH (but not in 0.1 M-KOH) is almost quantitatively transformed to guanine (Fig. 6a,b). In 0.1 M-KOH the u.v. spectrum of III slowly disappears; probably disruption of the purine base occurs.

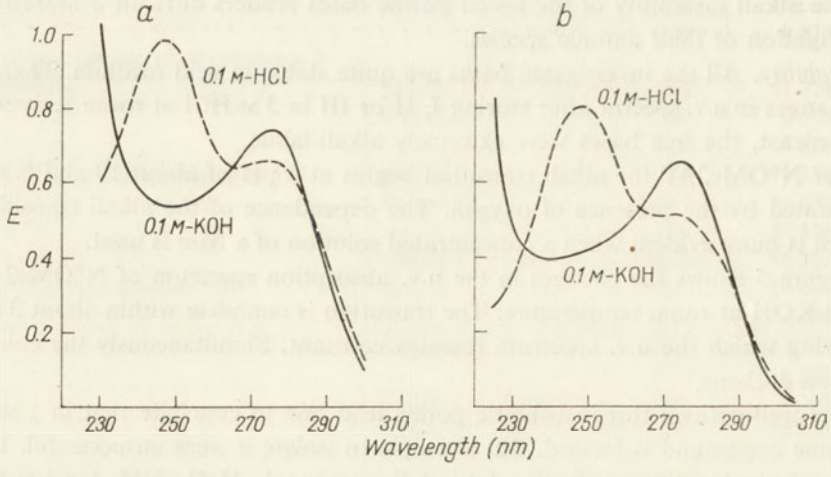


Fig. 6. The ultraviolet spectra of (*a*), N^6-hydroxy-2-aminopurine after transition in 1 M-KOH; (*b*), guanine. N^6-Hydroxy-2-aminopurine was kept in 1 M-KOH (3 h, room temperature) and aliquots removed and determined as indicated.

It has been noted that guanine, when sprayed on chromatograms with Pauly reagent, gives a characteristic orange spot. Since the orange spots were visible on chromatograms among the alkaline transition products of N^6MeOH2AP (II), and even N^6OMe2AP (I), it is possible that a small quantity of guanine is also formed during the further alkaline transformation of II and I.

There are several features common to the alkaline transformation of the above investigated bases, and N^6-hydroxypurine described by Giner-Sorolla: (i) glycosidation renders bases more stable against alkali transition, (ii) the reaction is oxygen (air) dependent.

For the N^6-hydroxypurine, formation of 6,6′-azoxypurine as the first product of alkali treatment was established (Giner-Sorolla *et al.*, 1966, 1970, 1972). The stabilizing effect of ribosidation on alkali transformation [ribosidation facilitates disruption of the imidazole part of purine (Gordon *et al.*, 1957)], and formation of guanine, suggests that in alkali the pyrimidine part and particularly the N^6-group are first affected. But there is no indication of 6,6′-azotype compound formation.

Synthesis of 5′-pyrophosphoriboside of N^6-methoxy-2-aminopurine and attempts at polymerization. The method of Moffatt & Khorana (1961), commonly applied to 5′-nucleoside phosphorylation, cannot be used in the case of N^6OMe2AP derivatives. Apparently morpholine reacts with the N^6OMe-substituent leading to loss of starting material. The methods of Michelson (1964) and Howard & Ott (1965) are both satisfactory. The latter, excellent for small-scale preparation, was used. This method was originally developed for the synthesis of 5′-triphosphate nucleosides but when tributylammonium monophosphate instead of tributylammonium pyrophosphate is used, the desired diphosphate nucleoside can be obtained.

The reaction mixture was then applied to Dowex HCO_3^- column and eluted with a gradient of H_2O (1.3 litres) - 0.3 M-triethylammonium saturated with CO_2, pH 7.5 (1.3 litres). The pyrophosphate fractions were pooled, freed of bicarbonate by several evaporations with methanol, and used for polymerization.

Attempts to synthesize the homopolymer of N^6OMe2AP were unsuccessful. Both *E. coli* and *M. luteus* polynucleoside phosphorylase were tested under a wide variety of conditions. These included: different concentrations of substrate, and different ratios of Mg^{2+} (or Mn^{2+}) to substrate; addition of ApA, or dithiothreitol. Sometimes slight traces of polymerization were seen, but these were insufficient for further purification.

When polymerization was conducted in the presence of N^6OMe2AP-riboside 5′-pyrophosphate and ADP, the copolymer of N^6OMe2AP,A, but with small yields: $\leqslant 10\%$, was obtained. The conditions used were as follows: 1.4 μmole of substrate (N^6OMe2AP-DP+ADP); $MgCl_2$, 5 - 6 mM; EDTA, 0.5 - 0.6 mM; NaN_3, 1.5 mM; Tris buffer, pH 8.5, 0.2 - 0.3 M and 15 μl of *M. luteus* enzyme (0.15 mg) per 90 μl of the above mixture.

After about 24 hours at 37°C, the copolymer was purified by deproteinization with neutral phenol, ether treatment, and dialysed against 0.1 M-Tris buffer, pH 7.4,

containing 0.1 M-NaCl and 1 mM-EDTA, then against a tenfold diluted salt-buffered mixture, and finally against water.

Only short chained polymers ($s_{20} \geqslant 2.0$) were obtained. The spectrum of the polymers depended on the ratio of N^{6}OMe2AP-DP: ADP used for polymerization, and is an indicator of N^{6}OMe2AP residue incorporation (Fig. 7).

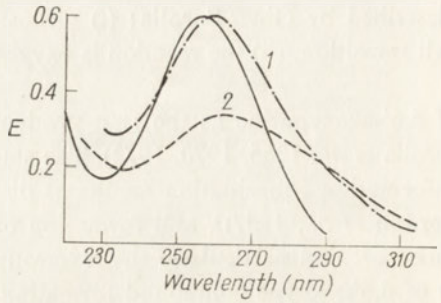


Fig. 7. The ultraviolet spectra in 0.01 M-phosphate buffer - 0.1 M-NaCl, pH 7.25, of poly A (—); and two different copolymers of N^{6}OMe2AP,A: (*1*), input ratio of N^{6}OMe2AP : ADP, 1 : 2.28; (*2*), input ratio of N^{6}OMe2AP : ADP, 1 : 0.65.

Neither of these copolymers forms complexes with poly(U). It appears that the presence of modified residues disrupts the structure of poly(A); the thermal hypochromicity is greatly reduced [7% for polymer product I as compared with 26% for poly(A)].

Mutagenic activity. Spot test analysis (Iyer & Szybalski, 1958) of *S. typhimurium* TA1530 strain (Ames, 1971) using 5×10^8 bacteria cells per Petri plate, and crystals (100 - 200 μg) of N^{6}OMe2AP (I), N^{6}MeOH2AP (II) or N^{6}OH2AP (III) revealed mutagenic activity of all tested compounds. Reversion of *his*$^- \rightarrow$ *his*$^+$ was determined. The order of mutagenicity was as follows: III $\geqslant$ II $\geqslant$ I. Both III and II (but not I) gave inhibition zones. NMeHA, OMeHA and HA in the same concentration range as the investigated purine analogues, did not show any mutagenic action. No mutagenic activity was observed with 2-aminopurine (2AP) and 5-bromodeoxyuridine (BudR).

DISCUSSION

When potential mutagens are tested in living systems, some doubts may arise as to whether their action occurs directly or *via* their metabolic product. Lack of reversion of *his*$^- \rightarrow$ *his*$^+$ mutation under the influence of HA, OMeHA or NMeHA eliminates any suspicion that these known chemical mutagens are released and are responsible for the observed biological effect. The reversion of the *his*$^- \rightarrow$ *his*$^+$ mutation in strain TA1530 can be induced by many alkylating agents such as diethylsulphate, methylethylsulphate, nitrogen mustard, *N*-methyl,*N'*-nitro-*N*-nitrosoguanidine. Lack of effect of 2AP and BudR and the high activity of alkylating agents known for their reaction mainly with guanine residues indicate that: (i) guanine is the main target for *his*$^- \rightarrow$ *his*$^+$ reversion, (ii) the newly investigated purine analogues act as analogues of guanine.

However, the mutagenic spectra of N^6OMe2AP, N^6MeOH2AP and N^6OH2AP seem to differ distinctly from those of BudR and 2AP, thus the former compounds can be useful in attempts to define the nature of the base undergoing mutational changes.

I should like to thank Mrs. Krystyna Myszkowska for excellent technical assistance and Mrs. Ewa Popowska for spot-test analysis.

REFERENCES

Ames, B.N. (1971) in *Chemical Mutagens. Principles and Methods for their Detection* (A. Hollaender, ed.) vol. 1, pp. 267 - 282. Plenum Press, New York - London.

Barner, H. & Cohen, S.S. (1959) *J. Biol. Chem.*, **234**, 2987 - 2991.

Freese, E.B. (1968) *Mutat. Res.*, **5**, 299 - 301.

Giner-Sorolla, A. (1970) *J. Heterocycl. Chem.*, **7**, 75 - 79.

Giner-Sorolla, A., O'Bryant, S.A., Burchenal, J.H. & Bendich, A. (1966) *Biochemistry*, **5**, 3057 - 3061.

Giner-Sorolla, A., O'Bryant S.A., Nanos, C., Dollinger, M.R., Bendich, A. & Burchenal, J.H. (1968) *J. Med. Chem.*, **11**, 521 - 523.

Giner-Sorolla, A., Burchenal, J.H. & Bendich, A. (1972) in *The Purines — Theory and Experiment*. Proceedings of an International Symposium Held in Jerusalem, 4 - 8 April 1971 (E.D. Bergmann & B. Pullman, eds.) pp. 478 - 503. Academic Press, New York.

Gordon, M.P., Weliky, V.S. & Brown, G.B. (1957) *J. Am. Chem. Soc.*, **79**, 3245 - 3250.

Howard, D.E. & Ott, D.G. (1965) *J. Am. Chem. Soc.*, **87**, 1785 - 1788.

Iyer, V.N. & Szybalski, W. (1958) *Appl. Microbiol.*, **6**, 23 - 29.

Janion, C. & Shugar, D. (1973) *Acta Biochim. Polon.*, **20**, 271 - 284.

Janion, C., Żmudzka, B. & Shugar, D. (1970) *Acta Biochim. Polon.*, **17**, 31 - 40.

Lane, B.G. (1963) *Biochim. Biophys. Acta*, **72**, 110 - 112.

Michelson, A.M. (1964) *Biochim. Biophys. Acta*, **91**, 1 - 13.

Moffatt, J.G. & Khorana, H.G. (1961) *J. Am. Chem. Soc.*, **83**, 649-658.

Sartorelli, A.C. & Upchurch, H.F. (1963) *Cancer Res.*, **23**, 1077 - 1084.

Sartorelli, A.C., Bieber, A.L., Chang, P.K. & Fischer, G.A. (1964) *Biochem. Pharmacol.*, **16**, 507 - 515.

Tabor, H. (1971) in *Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, eds.) vol. 3, p.631. Academic Press, New York.

Williams, F.R. & Grunberg-Manago, M. (1964) *Biochim. Biophys. Acta*, **89**, 66 - 89.

SYNTEZA I WŁAŚCIWOŚCI N^6-PODSTAWIONYCH POCHODNYCH 2-AMINOPURYNY

Streszczenie

1. Opisano syntezę, widma absorpcyjne w ultrafiolecie i zachowanie się w alkaliach N^6-metoksy-, N^6-metylo,hydroksy- i N^6-hydroksy-2-aminopuryn.

2. Przygotowano 5′-pirofosforan rybozydu N^6-metoksy-2-aminopuryny w celu jego polimeryzacji fosforylazą polinukleotydową.

3. Otrzymano kopolimer zawierający reszty rybozydu N^6-metoksy-2-aminopuryny i adenozyny; synteza homopolimeru nie powiodła się.

4. Stwierdzono, że wszystkie zsyntetyzowane analogi puryn wykazują działanie mutagenne na *Salmonella typhimurium* TA1530.

Received 31 October, 1975.

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 1

J. A. SIEDLECKI, RADOSŁAWA MIKKE and BARBARA ŻMUDZKA

DNA POLYMERASES OF MURINE LBN/b LEUKEMIC CELLS*

Institute of Oncology,
ul. Wawelska 15; 02-034 Warszawa, Poland

In the post-mitochondrial fraction of murine LBN/b leukemic cells, four fractions with DNA polymerase activity (I, II, III, IV) were found. On the basis of ion exchanger affinity and poly(A), poly(C) and poly(Cm) replication ability, fraction I was classified as RNA-directed DNA polymerase of viral origin. On the basis of the differences in the ion exchanger affinity, molecular weight, template requirement, pH-dependence of enzymatic activity and NaCl concentration, divalent ion requirements and susceptibility to *N*-ethylmaleimide inhibition, fractions II, III and IV were classified as DNA-directed DNA polymerases β, α and γ, respectively.

Three fractions, i.e. reverse transcriptase, and DNA-directed DNA polymerases β and γ, were found to incorporate dTMP on a poly(A) · oligo(dT) template-primer. Despite the similarity of the reaction of DNA polymerases β and γ with poly(A) · oligo(dT), some other properties of these enzymes suggest that they represent distinct enzymatic entities.

The number of DNA polymerases in normal and neoplastic animal cells, especially those incorporating deoxyribonucleoside triphosphates with RNA or polyribonucleotide as template, has not as yet been unequivocally determined, and is repeatedly subjected to verification using new biological models.

Apart from high-molecular DNA polymerase α (Bollum, 1975) and low-molecular DNA polymerase β (Chang, 1973), normal and neoplastic animal cells contain one, and according to some authors more than one, DNA polymerase γ exhibiting the poly(A) replication ability (Spadari & Weissbach, 1974a; Weissbach, 1975; Gerard, 1975). Cells infected with oncornavirus contain, in addition, reverse transcriptase (RNA-directed DNA polymerase). According to a number of authors, the ability to utilize poly(A) as template represents a test differentiating the two latter enzymes from DNA polymerases α and β (Fridlender *et al.*, 1972;

* This work was supported by the Polish Academy of Sciences within the project 09.3.1.

Lewis *et al.*, 1974; Srivastava, 1974). On the other hand, Chang has found that homogeneous DNA polymerase β obtained from bone marrow chromatin, as well as preparations of this enzyme isolated from chromatin of other animal tissues, were characterized by a high poly(A) replication activity (Chang, 1974). Because of this finding, Chang has questioned the existence of DNA polymerase γ as an enzyme independent of DNA polymerase α and β (Chang, 1974).

The present study comprises an analysis of DNA polymerases of murine LBN/b leukemic cells, with special consideration of those exhibiting poly(A) replication ability. LBN/b leukemia was initially induced by treatment of BN mice with antilymphocytic globulin, and then transplanted intraperitoneally; this procedure resulted in the development of ascitic tumour in all animals. The demonstration, in neoplastic cells, of the presence of C particles, the leukemia virus *gs-1* antigen and cell surface antigen related to infection with Murine Leukemia Virus-Gross suggested that the neoplastic process was induced by activation of the oncogenic virus rather than by the immunosuppressive action of antilymphocytic globulin (Szkudlarek *et al.*, 1976). Thus, it was expected that the LBN/b leukemic cells would be a source not only of DNA polymerases α, β and possibly γ, but also of reverse transcriptase derived from the virus.

MATERIALS AND METHODS

Substrates. Deoxynucleoside triphosphates were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). ^{3}H-labelled deoxynucleoside triphosphates were obtained from the Radiochemical Centre (Amersham, England). Specific activities are given with the experimental data.

Templates and primers. Native calf thymus DNA, purchased from Sigma, was converted to the activated form with DNase I according to the procedure of Fansler & Loeb (1974), dialysed against water, and stored at −28°C. Oligo(dT)$_{12-18}$, and oligo(dG)$_{12-18}$, were obtained from P-L Biochemicals, Inc. (Milwaukee, Wis., U.S.A.), and poly(A), poly(C) and poly(dT) from Miles Laboratories (Elkhart, Ind., U.S.A). Poly 2′-*O*-methylcytidylic acid was prepared according to a published procedure (Żmudzka *et al.*, 1969). Synthetic template-primer duplexes were prepared by annealing the two complementary homopolymers or homopolymer/oligomer at concentrations of 200 µg/ml in 5 mM-Tris-HCl buffer (pH 7.8) containing 10 mM-NaCl, at room temp. for 1 h and then at 4°C for 18 h.

Other materials and their sources were as follows: Whatman cellulose powder (CF 11) and DEAE-cellulose (DE-52), Serva phosphocellulose, Pharmacia Sephadex G-200 and CM-Sephadex (C-50 medium), Sigma calf thymus DNA, Worthington DNase I.

LBN/b leukemic cells. A transplantable line of leukemic LBN/b cells was obtained by treatment of mice (inbred strain BN/b) with antilymphocytic globulin D7 (Szkudlarek *et al.*, 1976). Transplantation by intraperitoneal inoculation of 30×10^6 cells into BN/b mice was lethal to 100% of animals within 10 days; however,

after 6 days it made available 800 mg wet mass of ascitic tumour cells per animal. The cells were harvested, washed with 0.9% NaCl, frozen and stored at —28°C.

Preparation of enzyme fractions. Frozen cells 30 g, were thawed at room temperature and suspended in 100 ml of cold 50 mM-Tris-HCl (pH 7.5) containing 1 mM-EDTA and 1 mM-DTT (buffer *A*). All subsequent steps were performed at 4°C. Cells were centrifuged at 1000 *g* for 10 min, resuspended, centrifuged once again in the same way and manually broken in a Potter homogenizer (twenty-five strokes). Microscopic examination showed that nearly all cells were disrupted. The homogenate was then centrifuged at 600 *g* for 10 min, whereupon the nuclear-membrane pellet was re-extracted with 10 ml of buffer *A* and centrifuged at 600 *g* for 10 min. The two supernatants were pooled and centrifuged at 15 000 *g* for 10 min. With constant slow stirring the 15 000 *g* supernatant was brought to a final concentration of 10% glycerol, 0.05% Triton X-100 and 1 M-KCl. After 60-min extraction with stirring, the material was centrifuged at 105 000 *g* for 2 h. The 105 000 *g* supernatant (92 ml), dialysed against 50 mM-Tris-HCl (pH 7.5) containing 1 mM-DTT and 10% glycerol (buffer *B*), is considered as the post-mitochondrial fraction.

Nucleic acid removal. The dialysed extract (A_{280}/A_{260} = 0.9 - 1.2) was diluted twofold with buffer *B* and loaded onto a DEAE-cellulose column (2 × 19 cm) equilibrated with the same buffer. The column was eluted with 0.25 M-KCl in buffer *B* until the effluent was free of all material absorbing at 280 nm (A_{280}/A_{260} = 1.7). The column flow-through and the 0.25 M-KCl wash were pooled and dialysed against buffer *B*.

DEAE-cellulose chromatography. The dialysed sample from the first DEAE-cellulose step was adsorbed on a DEAE-cellulose column (1.8 × 16 cm) equilibrated with buffer *B*. The column was washed with 0.5 M-KCl in buffer *B* until no more protein was eluted (detected by monitoring the $A_{280\ nm}$ of the effluent), and eluted with 0.25 M-KCl in buffer *B* (Fig. 1). The 0.25 M-KCl eluate absorbing at 280 nm was dialysed against buffer *B*.

Phosphocellulose chromatography. The flow-through and the 0.05 M-KCl eluate of the preceding DEAE-cellulose column were pooled and adsorbed on a phosphocellulose column (1.5 × 15 cm) equilibrated with 50 mM-Tris-HCl (pH 7.5) containing 1 mM-DTT, 0.1 mM-EDTA, 10% glycerol, 0.02% Triton X-100 and 0.1 mg/ml bovine serum albumin (buffer *C*). The column was washed with 20 ml of 0.075 M-KCl in buffer *C* and eluted with 200 ml of a linear gradient of 0.1 M to 0.8 M-KCl in buffer *C*. Fractions were assayed for polymerase activity as shown in Fig. 2. Fraction I was stored at —28°C, after 5 - 6-fold concentration in an Amicon standard stirred cell with UM 10 Diaflo membrane, and adjustment to a 50% glycerol concentration. Fraction II was stored at —28°C without concentration.

CM-Sephadex chromatography. The dialysed 0.25 M-KCl eluate of the DEAE-cellulose column was adsorbed on a CM-Sephadex column (1.4 × 25 cm) equilibrated with buffer *B*. The column was washed with the same buffer until free of material absorbing at 280 nm, and eluted with 120 ml of a linear gradient from 0.1 M to 0.7 M-KCl in buffer *B*. Fractions were assayed for polymerase activity as shown in Fig. 3. Fractions III and IV, respectively, were concentrated several times in an Amicon

standard stirred cell with UM 10 Diaflo membrane, dialysed, adjusted to a 50% glycerol concentration and stored at −28°C.

DNA-cellulose chromatography. Single-stranded calf thymus DNA-cellulose was prepared according to the method of Alberts & Herrick (1971). A 50-µl sample of the concentrated and dialysed fraction IV from CM-Sephadex was adsorbed for 60 min on a DNA-cellulose column (0.6 × 4 cm) equilibrated with 0.01 M-Tris-HCl (pH 7.5) containing 0.05 M-KCl, 1 mM-mercaptoethanol, 20% glycerol and 0.1 mg/ml bovine serum albumin (buffer *D*). The column was washed with 5 ml of buffer *D* and eluted with 8 ml of a linear gradient from 0.075 M to 0.80 M-KCl in buffer *D*. The flow rate of the column was adjusted to about 0.02 ml per minute. Fractions were assayed for polymerase activity as shown in Fig. 4.

Preparation of DNA polymerase fractions I and II from microsomes. Frozen LBN/b cells (10 g) were thawed, suspended in 30 ml of 50 mM-Tris-HCl (pH 7.5), 0.5 mM-EDTA, 1 mM-mercaptoethanol, 5 mM-$MgCl_2$ and 0.25 M-sucrose (buffer *F*), and then homogenized manually. The homogenate was centrifuged at 900 *g* for 10 min, and the resulting supernatant was centrifuged at 10 000 *g* for 15 min. The 10 000 *g* supernatant was centrifuged through a cushion of 25% (w/w) sucrose in buffer *F* at 160 000 *g* for 2 h. The 160 000 *g* pellet was suspended in buffer containing 1 M-KCl and 0.5% Triton X-100, and extracted with stirring for 1 h. The mixture was centrifuged at 170 000 *g* for 1 h, and the supernatant was dialysed and separated from nucleic acids on a DEAE-cellulose column (1.5 × 10 cm), as described for the post-mitochondrial fraction. Protein eluted with 0.35 M-KCl were subjected to a two-fold concentration in an Amicon standard stirred cell with UM10 Diaflo membrane, dialysed and loaded onto a DEAE-cellulose column (1.5 × 10 cm).The column flow-through and the 0.075 M-KCl (in buffer *B*) wash were loaded onto a phosphocellulose column (1 × 11 cm), which was washed with 7 ml of 0.075 M-KCl in buffer *C* and eluted with 80 ml of linear gradient from 0.1 to 0.75 M-KCl in buffer *C*. DNA polymerase activity of the effluent fractions was assayed as described in Fig. 2.

Sucrose gradient centrifugation. From 2% to 22% linear sucrose gradients (5 ml) in 50 mM-Tris-HCl (pH 7.5) containing 0.2 M-KCl, 1 mM-mercaptoethanol, 1 mM-EDTA (buffer *E*), were prepared. Samples (0.1 ml) of fraction II from phosphocellulose, and of fractions III and IV from CM-Sephadex, were dialysed against buffer *E* and separately applied on the gradient; they were then centrifuged at 64 000 r.p.m. for 5 h in the SW65L rotor of a Beckman model L5-65 centrifuge. Gradients were fractionated with the aid of the Beckman fraction recovery system into 40 equal fractions, from the bottom of the tube. Fractions were assayed for biological activity as shown in Fig. 5.

Gel filtration on Sephadex G-200. A 1-ml sample of fraction IV from CM-Sephadex was loaded onto a Sephadex G-200 column (1.9 × 70 cm) equilibrated with 50 mM-Tris (pH 7.9) containing 0.2 M-KCl, 1 mM-DTT, 1 mM-EDTA and 10% glycerol. The material was eluted with the same buffer and assayed for polymerase activity as shown in Fig. 6. The flow rate of the buffer was 3 ml/h·cm² of the gel surface; 1.2 ml fractions were collected.

DNA polymerase assays. DNA polymerase activity was assayed using either activated calf thymus DNA (*system A*) or synthetic homopolymer primer templates (*system B*). In *system A* the standard reaction mixture contained, in a final volume of 50 µl, the amount of enzyme as indicated in each experiment, 40 mM-potassium phosphate (pH 7.0), 0.07 M-KCl, 8 mM-$MgCl_2$, 0.8 mM-DTT, 1 mg/ml bovine serum albumin, 300 µg/ml activated calf thymus DNA, 100 µM each of dCTP, dATP and dGTP, and 100 µM-[^{3}H]dTTP (200 cpm/pmole), unless otherwise stated. In *system B* the reaction mixture contained, in a final volume of 50 µl, the amount of enzyme as indicated in each experiment, 40 mM-Tris-HCl (pH 8.0), 0.07 M-KCl, 0.4 mM-$MnCl_2$, 1.6 mM-DTT, 1 mg/ml bovine serum albumin, 20 µg/ml poly(A) with 10 µg/ml oligo$(dT)_{12-18}$ and 100 µM-[^{3}H]dTTP (200 cpm/pmole), unless otherwise stated. If 20 µg poly(dA) with 1 µg oligo$(dT)_{12-18}$ or 20 µg poly(C) and 20 µg poly(Cm) with 20 µg oligo$(dG)_{12-18}$ were used as templates, then 0.5 µM-[^{3}H]dTTP (8000 cmp/pmole) or 0.5 µM-[^{3}H]dGTP (8000 cpm/pmole) were used as substrates. Incubations were at 37° for 60 min. Any deviations from standard conditions and procedure are noted in the descriptions of the individual experiments. Acid-precipitable radioactivity was collected and washed on Whatman no. 1 filters or glass fiber (Whatman GF/C) by the technique of Bollum (1966). One unit of enzymatic activity is defined as 1 nmole of radioactive deoxynucleotide incorporated into the acid-precipitable material in standard *system A*, at 37°C per 60 min. Protein determinations were performed by the method of Lowry *et al.* (1951).

RESULTS

Isolation of DNA polymerases. The present investigations on DNA polymerases of murine LBN/b leukemic cells were limited to the cytoplasmatic post-mitochondrial fraction since this fraction contains all types of DNA polymerases, including those utilizing ribose templates. The cytoplasmic enzymes were solubilized by extraction with detergent and KCl, separated from nucleic acids on DEAE-cellulose, and then isolated as four fractions (I, II, III, IV) regarded as reverse transcriptase, and DNA-directed DNA polymerases β, α and γ, respectively. The separation was based on differences in the ion exchanger affinity of the individual polymerases applied by Lewis *et al.* (1974) to the development of a technique for isolation and separation of DNA polymerases of mammalian tissues infected with oncogenic RNA virus.

With the above-mentioned technique (Lewis *et al.*, 1974) reverse transcriptase and DNA polymerase β were isolated as fractions not adsorbed on DEAE-cellulose in the presence of 0.05 - 0.075 M-KCl (Fig. 1), and separated on phosphocellulose into two fractions (I and II, respectively) (Fig. 2A).

Reverse transcriptase was also obtained from the microsomal fraction, initially isolated from the post-mitochondrial fraction. The detailed preparative treatment is described under Methods. It can be seen from Fig. 2B that the microsomal extract exhibited a more advantageous fraction I/fraction II ratio, as compared with the

post-mitochondrial fraction extract. Moreover, preliminary concentration of the microsomes increased the total activity of fraction I. From tests applied in this study, there were no differences between the reverse transcriptase preparations obtained from both sources.

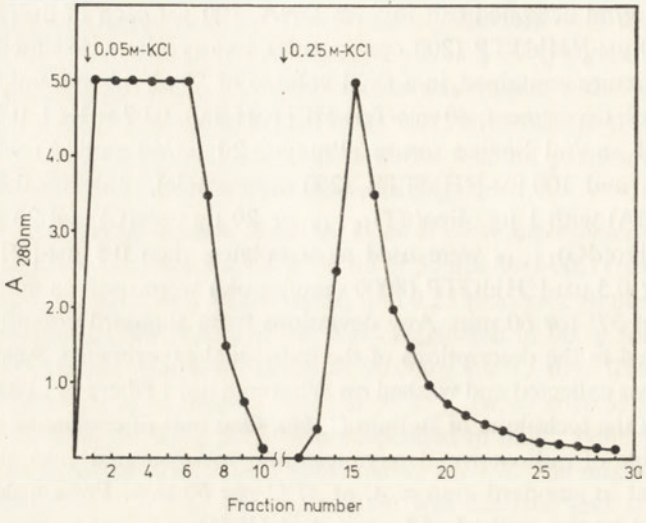


Fig. 1. DEAE-cellulose chromatography of DNA polymerases from the post-mitochondrial fraction of LBN/b cells. Fractions of 10 ml were collected on elution with 0.05 M-KCl, and of 3 ml on elution with 0.25 M-KCl. For details see Methods.

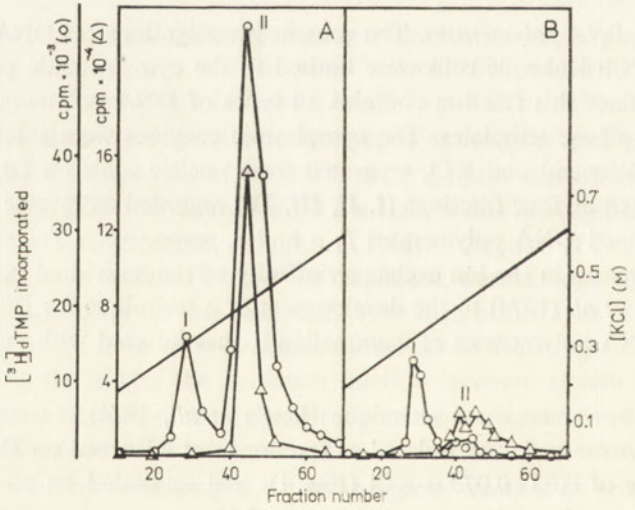


Fig. 2. Phosphocellulose chromatography of the 0.05 M-KCl and 0.075 M-KCl DEAE-cellulose wash obtained, respectively, from the post-mitochondrial (*A*) and microsomal (*B*) fractions of LBN/b cells. For details of preparation and chromatography see Materials and Methods. 10-µl samples of the effluent fractions were assayed under standard conditions with $(A)_n \cdot (dT)_{12-18}$ (○) and $(dA)_n \cdot (dT)_{12-18}$ (△) as templates and 2.5 µM (*A*) and 5 µM (*B*) [^{3}H]dTTP (8000 cpm/pmole) as substrate. No potassium chloride was added to the reaction mixture.

The biologically active material adsorbed on DEAE-cellulose and eluted with 0.25 M-KCl (Fig. 1) contained two further DNA polymerases (α and γ) which were separated on CM-Sephadex as fractions III and IV (Fig. 3). In some experiments, the fraction of polymerase α partially separated into two peaks showing no difference in the biological activity; this phenomenon could be a reflection of the heterogeneity of this fraction with respect to molecular weight. In addition, in some experiments a small peak appeared at 0.7 M-KCl which incorporated dTMP with poly(A)·oligo(dT) template but had not been further analysed.

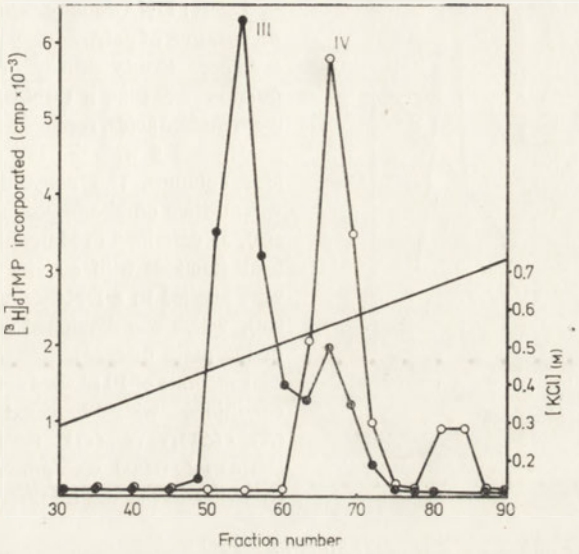


Fig. 3. CM-Sephadex chromatography of the 0.25 M-KCl DEAE-cellulose eluate obtained from the post-mitochondrial fraction of LBN/b cells; the eluate was treated as described in Materials and Methods, and 5 µl samples were assayed under standard conditions with the activated DNA (●) and $(A)_n \cdot (dT)_{12-18}$ (○) as templates, and 0.5 µM-[^{3}H]dTTP (8000 cpm/pmole) as substrate.

The application of CM-Sephadex chromatography to polymerases α and γ, instead of phosphocellulose chromatography used at this stage by Lewis *et al.*, proved to be preferable, since it improved the yield (from 20% to 60%, as calculated from the amount of enzyme in the crude post-mitochondrial fraction), and — moreover — permitted separation of both polymerases at this stage. The latter fact was testified to by the inactivity of a polymerase preparation thus isolated on a $(A)_n \cdot (dT)_{12-18}$ template, as well as by the result of polymerase γ chromatography on denatured DNA-cellulose, presented in Fig. 4. According to Fig. 4, the preparation of DNA polymerase γ isolated on CM-Sephadex was homogeneous, whereas that prepared on phosphocellulose remained a mixture which only separated into polymerases α and γ on denatured DNA-cellulose when eluted with 0.32 and 0.56 M-KCl, respectively (Fig. 4, Insert). Attempts at further purification of polymerase γ by filtration on Sephadex G-200 or centrifugation in a sucrose gradient resulted, as in the case of DNA polymerase β, in an abrupt drop in activity, especially activity toward synthetic templates.

Properties of reverse transcriptase, as well as of DNA polymerases β, α and γ, were studied using concentrated, dialysed fractions I and II obtained from phosphocellulose (Fig. 2), and fractions III and IV from CM-Sephadex (Fig. 3), all stored at −28°C.

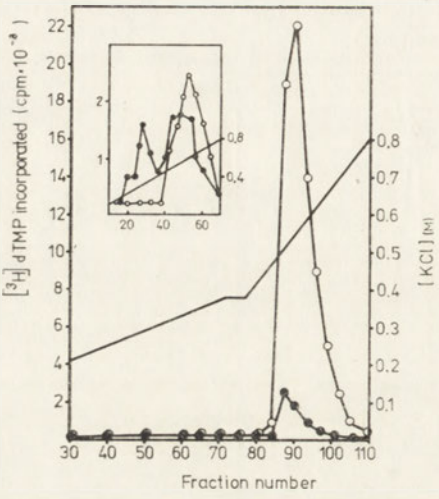


Fig. 4. DNA-cellulose affinity chromatography of DNA polymerase fraction IV from CM-Sephadex. Fraction IV (0.05 enzyme unit) was adsorbed and eluted as described in Materials and Methods.

Insert: DNA-cellulose chromatography of the preparation of polymerase γ purified on phosphocellulose. Crude extract of post-mitochondrial fraction from 28 g LBN/b cells was separated from nucleic acids, reverse transcriptase and DNA polymerase β with the use of two DEAE-cellulose columns. The dialysed 0.25 M-KCl eluate was purified on phosphocellulose column (1.5 × 15 cm), as described in Materials and Methods, and peak fractions with activity on $(A)_n \cdot (dT)_{12-18}$ were applied to a DNA-cellulose column (0.6 × 4 cm), which was developed as the column shown in the main diagram.

Fractions of 10 µl were assayed under standard conditions with activated DNA (●) and $(A)_n \cdot (dT)_{12-18}$ (○) templates and 1 µM-[^{3}H]dTTP (8000 cpm/pmole) as substrate. Salt concentration in the reaction mixture was 0.1 M.

Sedimentation constant and molecular weight. The sedimentation constants of the enzymes present in fractions II, III and IV were determined by sedimentation in a sucrose density gradient (Fig. 5), using lysozyme, concavalin A and catalase as standards, with sedimentation constants of 2.1, 3.9 and 11.3, respectively. The values of the sedimentation constants, measured with an accuracy of ±10%, were 3.0, 7.5 - 9.5, and 3.1, respectively. The molecular weights, calculated according to Martin & Ames (1961) from the sedimentation constants, were 37 000, 130 000 - 200 000 and 40 000, respectively. The molecular weight of the enzyme in fraction IV determined by Sephadex G-200 filtration (Fig. 6), in the presence of cytochrome *c*, lysozyme, ovalbumin, bovine serum albumin and catalase as standards (with molecular weights of 12 000; 17 000; 45 000; 69 000 and 250 000, respectively), measured with an accuracy of ±15%, amounted to 50 000. The values of the molecular weight of the enzyme in fraction IV, determined by both methods, were consistent within the limits of experimental error. All measurements of molecular weights were carried out in 0.2 M-NaCl; in the absence of NaCl, aggregation of the enzymes occurred.

Effect of NaCl. Enzymatic fractions II, III and IV were characterized by differences in the effect of NaCl concentration on the DNA replication activity. Fraction III exhibited maximum activity when no NaCl was added to the reaction medium; in the presence of 0.1 M-NaCl it showed only 8% of the activity found under optimum conditions (Fig. 7). By contrast, fractions II and IV were clearly stimulated by NaCl,

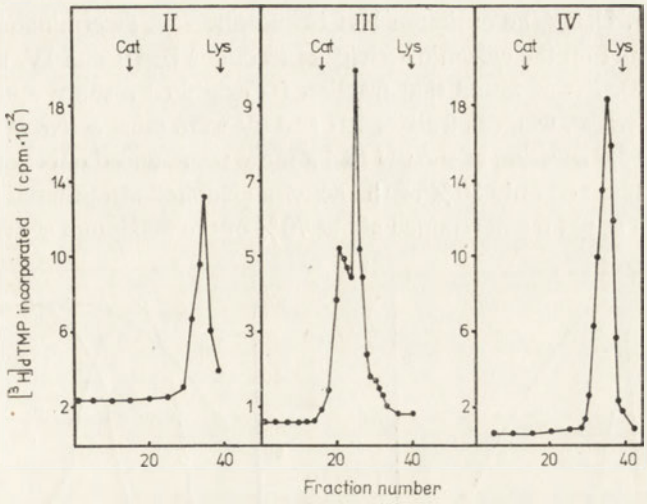
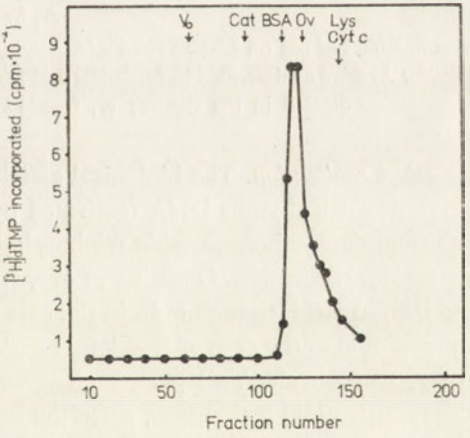


Fig. 5. Sucrose gradient centrifugation of LBN/b cell DNA polymerase fractions II, III and IV. 0.1-ml solutions of fractions II, III and IV containing 0.4, 1.5 and 0.2 enzyme units, respectively, were centrifuged and fractionated as indicated in Materials and Methods. Fractions were tested for DNA polymerase activity: fraction II in *system B* with 15 µl of gradient fraction and 2.5 µM-[^{3}H]dTTP (8000 cmp/pmole); fraction III in *system A*, with 5 µl of gradient fraction and 0.5 µM-[^{3}H]dTTP (8000 cmp/pmole), fraction IV in *system B* with $(dA)_n \cdot (dT)_{12-18}$ template instead of $(A)_n \cdot (dT)_{12-18}$, 25 µl of gradient fraction and 0.5 µM-[^{3}H]dTTP (8000 cpm/pmole). NaCl was omitted in all assays. Sucrose concentration was measured refractometrically. Marker proteins, lysozyme and catalase, were centrifuged and determined as described in Fig. 6.

Fig. 6. Sephadex G-200 gel filtration of LBN/b cell DNA polymerase fraction IV. One-ml sample (0.2 enzyme unit) of fraction IV from the CM-Sephadex column was loaded onto a Sephadex G-200 column and eluted as described in Materials and Methods. DNA polymerase activity of the fractions was assayed in standard *system B* with $(dA)_n \cdot (dT)_{12-18}$ instead of $(A)_n \cdot (dT)_{12-18}$, 15 µl of column fraction and 1 µM-[^{3}H]dTTP (8000 cpm /pmole). No NaCl was added to the reaction mixture. Marker proteins were chromatographed individually on the same column, and cytochrome *c* (Cyt. *c*), lysozyme (Lys), ovoalbumin (OV), and bovine serum albumin (BSA) were determined by absorption at 280 nm. Catalase (Cat) was determined by biological assay with H_2O_2. V_0 is the void volume measured with the use of dextran blue.



exhibiting maximum activity at an NaCl concentration of about 75 mM. For fraction IV the range of stimulating concentrations was narrow; for example, in 0.2 M-NaCl the replication activity decreased to 35% of the maximum. On the other hand, fraction II retained this activity also at higher NaCl concentrations (Fig. 7).

Effect of pH. Using the optimum NaCl concentration, determination was made of the effect of pH on the enzymic activity of fractions II, III and IV, using a DNA template (Fig. 8). It was found that fraction III exhibited a sharp optimum within the neutral pH range, whereas fractions II and IV were most active in alkaline medium, at pH 8 - 9. Lowering of the pH had a more pronounced effect on fraction II, which at pH 7 retained only 30% of the activity exhibited at optimum pH, whereas fraction IV even at pH 6 still retained about 70% of the maximum activity.

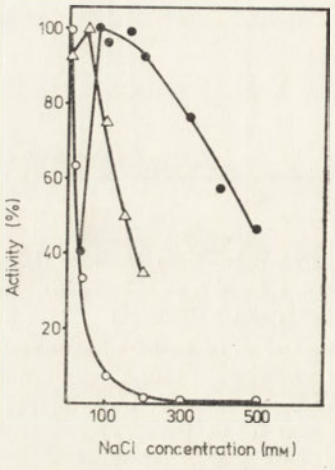


Fig. 7

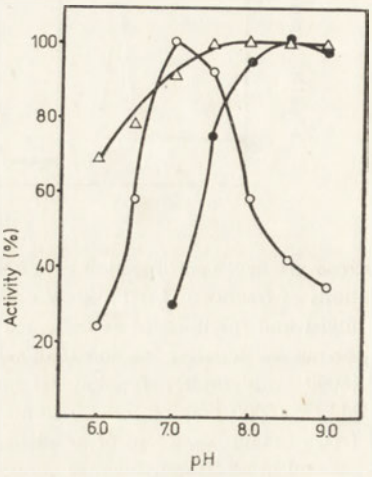


Fig. 8

Fig. 7. Effect of NaCl concentration on LBN/b cell DNA polymerase fractions II, III and IV. Incubation mixtures were the same as those referred to in Materials and Methods as *system A* assay, except for the presence of the indicated concentrations of NaCl; ●, fraction II; ○, fraction III; △, fraction IV.

Fig. 8. Effect of pH on LBN/b cell DNA polymerase fractions II, III and IV. Incubation mixtures (*system A*) were as described in Materials and Methods, except for the pH values, which were as indicated in the Figure; ●, fraction II; ○, fraction III; △, fraction IV.

DNA replication. The biological activity of fractions II, III and IV was determined using calf thymus DNA (activated by controlled hydrolysis with DNase I) and the four deoxyribonucleoside triphosphates, whose presence was indispensable for maximum replication. Omitting of one, two or three triphosphates reduced the polymerization yield, though even in the case of only one substrate this yield amounted, for example in the case of fraction II and IV, to 60% and 27% of the maximum activity, respectively. This phenomenon was not due to concentration with terminal deoxynucleotidyl transferase, since the occurrence of this enzyme was ruled out by the absence of substrate incorporation upon use of either oligo$(dT)_{12-18}$ or oligo $(dA)_6$ as the only reaction primer. The reliability of the above test was verified with the use of terminal transferase isolated from calf thymus (Bollum *et al.*, 1974), with either oligonucleotide or activated DNA as primer. Thus, it seems probable that the intense substrate incorporation, observed both in this study and by other authors (Chang & Bollum, 1972) in the reaction of eucaryotic DNA polymerase with acti-

vated DNA and one deoxynucleoside triphosphate, can be a reflection of addition to the large number of free 3'-OH ends occurring in activated DNA (Sedwick *et al.*, 1972).

K_m of thymidine 5'-triphosphate. The effect of dTTP concentration on the DNA replication rate under optimal conditions of pH and NaCl concentration is illustrated in Fig. 9. The values of K_m with dTTP, obtained from the Lineweaver-Burk plot, amounted for fraction II, III and IV to 20.0, 11.1 and 0.59 μM, respectively. The striking difference in the K_m value between fraction IV on the one hand, and fractions II and III on the other, resembles the results of Spadari & Weissbach (1974a), who found that the K_m values of RNA-directed DNA polymerases isolated from the nucleus and cytoplasm of HeLa cells are by more than one order of magnitude lower than the K_m values of DNA-directed DNA polymerase obtained from the same cells.

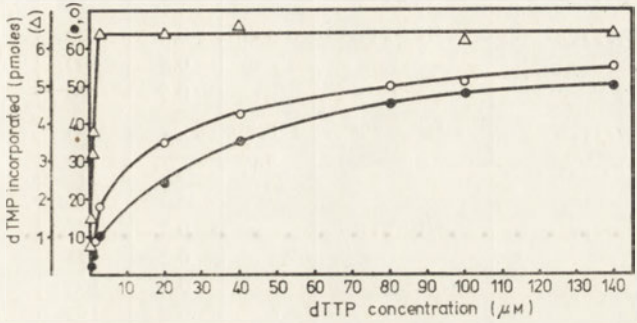


Fig. 9. Effect of substrate (dTTP) concentration on the rate of polymerization carried out with LBN/b cell DNA polymerase fractions II, III and IV. Reaction rate was measured in standard *system A* with 10 μl fraction II (●), 2 μl fraction III (○), 2 μl fraction IV (△). [^{3}H]dTTP (8000 cpm/pmole) was combined with a dTTP solution to concentrations given in the Figure.

Susceptibility to N-ethylmaleimide. Addition to the reaction mixture of *N*-ethylmaleimide to a final concentration of 1 and 10 mM, respectively, caused a change in the activated DNA replication yield; in the case of fractions II, III and IV this yield was 105 and 100%, 25 and 10%, and 50 and 90%, respectively, of the activity in the absence of *N*-ethylmaleimide.

Polyribonucleotide replication. Data presented in Figs. 2 and 3 pointed to the presence, in the material, of three enzymic fractions (I, II and IV) polymerizing dTTP on $(A)_n \cdot (dT)_{12-18}$ template. The highest activity was found with fraction IV. The replicative nature of the process catalysed by this enzyme was confirmed by the absence of reaction either with [^{3}H]dGTP as the only substrate, or after hydrolysis of poly(A) during preincubation with 0.4 M-KOH at 37°C for 60 min (unpublished). According to the data from Table 1, the $(A)_n \cdot (dT)_{12-18}$ replication required the presence of divalent cations in the incubation mixture, and attained a maximum yield in the presence of 0.2 mM-$MnCl_2$. As for DNA replication (Fig. 7), the enzymic activity of fraction IV was stimulated by addition of NaCl, with maximum activity found in the presence of about 100 mM NaCl. Fraction IV was stabilized by addition of dithiothreitol and albumin to the reaction medium.

Table 1

Characteristics of poly(A)-directed DNA synthesis in the presence of DNA polymerase γ from the post-mitochondrial fraction of LBN/b cells

The reaction was performed as described in Materials and Methods, with 0.5 μM-[^{3}H]dTTP (8000 cmp/pmole). DNA polymerase activity under the experimental conditions is expressed relative to the activity exhibited in the presence of activated DNA under optimum conditions.

Template	Substrates	Mg^{2+} (mM)	Mn^{2+} (mM)	Na^+ (mM)	Activity (%)
Activated DNA	^{3}H-dTTP, dATP, dCTP, dGTP	8.0	—	—	100
Activated DNA	^{3}H-dTTP	8.0	—	—	27
$(A)_n \cdot (dT)_{12-18}$	^{3}H-dTTP	—	—	100	0.9
$(A)_n \cdot (dT)_{12-18}$	^{3}H-dTTP	—	0.2	100	500
		—	0.3	100	183
		—	0.5	100	18
$(A)_n \cdot (dT)_{12-18}$	^{3}H-dTTP	0.4	—	100	2.1
		4.0	—	100	9.0
		40.0	—	100	3.1
$(A)_n \cdot (dT)_{12-18}$	^{3}H-dTTP	—	0.2	—	36
		—	0.2	50	320
		—	0.2	100	500
		—	0.2	150	430
		—	0.2	200	267

Fraction I (derived from the microsomal or post-mitochondrial fraction), another $(A)_n \cdot (dT)_{12-18}$ replicating enzyme, was the only one exhibiting dGMP incorporation ability, irrespective whether $(C)_n$ or $(Cm)_n$ — complexed with $(dG)_{12-18}$ — were used as templates. There were no significant differences between the reactions of fraction I with $(C)_n$ or $(Cm)_n$, these reactions, however, gave a fourfold lower yield, as compared with the reaction in which $(A)_n \cdot (dT)_{12-18}$ was used as template and dTTP and dGTP were at equal concentrations. A similar activity ratio on $(A)_n$, $(C)_n$ and $(Cm)_n$ templates was reported by Baltimore & Smoler (1971) for Moloney mouse leukemia virus, Gerard *et al.* (1974) for the murine Harvey strain of sarcoma leukemia virus and by Abrell & Gallo (1973) for Rauscher leukemia virus. On the other hand, in the case of reverse transcriptase preparations derived from viruses of other animals, e.g. from primate (Abrell & Gallo, 1973) and avian (Baltimore & Smoler, 1971) viruses as well as isolated from human neoplastic cells (Gerard *et al.*, 1975), poly(C) and poly(A) were found to be equivalent templates.

The further peak exhibiting $(A)_n \cdot (dT)_{12-18}$ replication activity coincided with fraction II. According to Fig. 2, fraction II also exhibited the activity with poly $(dA) \cdot oligo(dT)_{12-18}$, thus clearly differing from fraction I which showed no such activity.

The ability of polymerases β and γ to incorporate dTMP on synthetic templates disappeared more rapidly than the ability to incorporate dTMP on activated DNA.

The loss of activity took place upon enzyme centrifugation in a sucrose gradient, Sephadex G-200 filtration, concentration and storage, as well as during thermal inactivation. The present results are not adequate for an interpretation of these facts.

DISCUSSION

According to the present results, the cytoplasm of the murine LBN/b leukemia cells contains four enzymes polymerizing deoxyribonucleoside triphosphates. Two of them, corresponding to fractions III and II, were, on the basis of their ion exchanger affinity and molecular weights (130 000 - 200 000 and 37 000, respectively), classified as the two main DNA-dependent DNA polymerases α and β. The facts that: 1) NaCl inhibits polymerase α and stimulates polymerase β; 2) polymerase α exhibits maximum activity in neutral medium and polymerase β — in alkaline medium; and 3) *N*-ethylmaleimide inhibits only polymerase α, support our assignment, since these properties are exhibited by pure preparations of polymerases α and β (Bollum, 1975).

The suggestion that fraction I can contain reverse transcriptase is based on the following findings: 1) fraction I is eluted from DEAE-cellulose and phosphocellulose at the same NaCl concentrations and in the same position relative to DNA polymerases α, β and γ as the reverse transcriptase from human lymphoblasts infected with Simian Sarcoma Virus (Lewis *et al.*, 1974); and 2) fraction I is able to incorporate dGMP in the presence of poly(C) and poly(Cm) templates. Replication of poly(C)·oligo$(dG)_{12-18}$ has been for a long time assumed to be a property differentiating reverse transcriptase of viral origin from cellular DNA polymerase γ replicating poly(A)·oligo$(dT)_{12-18}$ (Lewis *et al.*, 1974; Mondal *et al.*, 1975). However, Spadari & Weissbach (1974a) have demonstrated that in the case of DNA polymerase γ of HeLa cells, isolated on DEAE-cellulose and phosphocellulose, the lack of ability to replicate $(C)_n \cdot (dG)_{12-18}$ and other polyribonucleotides was transient and disappeared on further purification of the enzyme on hydroxyapatite. However, the enzyme thus purified failed to replicate $(Cm)_n \cdot (dG)_{12-18}$ (Gerard, 1975) which for the reverse transcriptase of avian and murine viruses (Gerard *et al.*, 1974), as well as of human neoplastic cells (Gerard *et al.*, 1975), is as good a template as $(C)_n \cdot (dG)_{12-18}$. The enzyme present in fraction I was able to use both polynucleotides, poly(C) and poly(Cm), as templates.

The fourth enzyme isolated in this investigation exhibited the properties of DNA polymerase γ. In addition to DNA replication, it actively replicated poly(A) complexed with oligo(dT) or poly(dT); in this reaction it was characterized by an absolute requirement for Mn^{2+}, as well as by an optimum NaCl concentration and pH typical for DNA polymerase γ from HeLa cells (Fridlender *et al.*, 1972) and other tissues (Bolden *et al.*, 1972; Livingston *et al.*, 1974; Srivastava, 1974). The values of the molecular weight (40 000) and of the sedimentation constant (3.1S) found for the polymerase in this study, although not markedly different from the value of 50 000 reported by Maia *et al.* (1971), are significantly lower than the value

of 110 000 found by Spadari & Weissbach (1974a), and the value of 6 - 7S reported by Srivastava (1974). Differences in sedimentation constants of DNA polymerase γ, depending on the origin of the enzyme, were earlier noted by Lewis *et al.* (1974) who obtained DNA polymerase preparations with sedimentation constants of 6.3, 6.1 and 5.4S. The source of these differences remains unclear.

In addition, the problem of the number of poly(A)-replicating DNA polymerases in the cytoplasm, as well as the relationship between polymerases β and γ, deserve mention. According to the present findings, the ability to synthesize poly(dT) on an $(A)_n \cdot (dT)_{12-18}$ template is exhibited not only by DNA polymerase γ but also by the main polymerase fraction non-adsorbable on DEAE-cellulose, i.e. by polymerase β. This result is inconsistent with the data of many authors (Sedwick *et al.*, 1972; Fridlender *et al.*, 1972; Bolden *et al.*, 1972; Lewis *et al.*, 1974; Srivastava, 1974; Wang *et al.*, 1974) who have not found this activity in DNA polymerase β derived from various biological sources and separated, as in this study, from DNA polymerases α and γ by the ion exchanger chromatography. On the other hand, Chang (1974) has demonstrated that homogeneous DNA polymerase β from rabbit bone marrow chromatin, and from chromatin of other mammalian tissues, replicate $(A)_n \cdot (dT)_{12-18}$ with high fidelity. The negative reaction of DNA polymerase β with $(A)_n \cdot (dT)_{12-18}$, described by other authors, can be due, according to Chang, to differences in the experimental conditions or to the presence of nucleases in DNA polymerase β preparations (Chang, 1974). The demonstration, by Chang, of the poly(A) replication ability of DNA polymerase supports the present findings suggesting the possibility of isolation of as many as three enzymic fractions synthesizing poly(dT) on poly(A) template (i.e. reverse transcriptase, and DNA polymerases β and γ). On the other hand, the relationship between these activities is not clear.

These observations led Chang (1974) to question the distinct nature of DNA polymerases β and γ. According to this author, the chromatographic differences between these two enzymes can reflect dimerization of the former enzyme or incomplete separation of the latter from nucleic acids or other proteins. Consequently, comparison was made, in this aspect, of the properties of DNA polymerases β and γ, isolated in this study. These enzymes were found to differ in the sensitivity to pH, NaCl, *N*-ethylmaleimide and, above all, they differ by more than one order of magnitude in the values of the Michaelis constants. Moreover, the differences in the behaviour of DNA polymerases β and γ during the life cycle of the cell merit comment. According to Spadari & Weissbach (1974b), in synchronized cultures of HeLa cells the level of DNA polymerase β remains constant, whereas that of DNA polymerase γ increases in the early period of phases. It was consequently suggested that, although further comparative studies on the properties of these two enzymes are necessary, the available evidence favours the existence of two distinct activities, i.e. polymerases β and γ.

We thank Prof.Dr. A. Michałowski and Prof. Dr. D. Shugar for critical reading of the manuscript, Dr. A. Czarnomska for LBN/b cells and Miss E. Poddany for excellent technical assistance.

REFERENCES

Abrell, J.W. & Gallo, R.C. (1973) *J. Virol.*, **12**, 431 - 439.

Alberts, B. & Herrick, G. (1971) in *Methods in Enzymology* (L. Grossman & K. Moldave, eds.) vol. 21, D, pp. 198 - 217. Academic Press, New York - London.

Baltimore, D. & Smoler, D. (1971) *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1507 - 1511.

Bolden, A., Fry, M., Muller, R., Citarella, R. & Weissbach, A. (1972) *Arch. Biochem. Biophys.*, **153**, 26 - 33.

Bollum, F.J. (1966) in *Procedures in Nucleic Acid Research* (G.L. Cantoni & D.R. Davies, eds.) pp. 296 - 300. Harper & Row, New York.

Bollum, F.J. (1975) in *Progress in Nucleic Acid Research and Molecular Biology* (W.E. Cohn, ed.) vol. 15, pp. 109 - 144. Academic Press, New York - San Francisco - London.

Bollum, F.J., Chang, L.M.S., Tsiapalis, C.M. & Dorson, J.W. (1974) in *Methods in Enzymology* (L. Grossman & K. Moldave, eds.) vol. 29, E, pp. 70 - 81. Academic Press, New York - London.

Chang, L.M.S. (1973) *J. Biol. Chem.*, **248**, 3789 - 3795.

Chang, L.M.S. (1974) *J. Biol. Chem.*, **249**, 7441 - 7446.

Chang, L.M.S. & Bollum, F.J. (1972) *Biochemistry*, **11**, 1264 - 1272.

Fansler, B.S. & Loeb, L.A. (1974) in *Methods in Enzymology* (L. Grossman & K. Moldave, eds.) vol. 29, E, pp. 53 - 70. Academic Press, New York - London.

Fridlender, B., Fry, M., Bolden, A. & Weissbach, A. (1972) *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 452 - 455.

Gerard, G.F. (1975) *Biochem. Biophys. Res. Commun.*, **63**, 706 - 711.

Gerard, G.F., Rottman, F. & Green, M. (1974) *Biochemistry*, **13**, 1632 - 1641.

Gerard, G.F., Loewenstein, P.M., Green M. & Rottman, F. (1975) *Nature* (*London*), **256**, 140 - 143.

Lewis, B.J., Abrell, J.W., Smith, R.G. & Gallo, R.C. (1974) *Biochim. Biophys. Acta*, **349**, 148 - 160.

Livingston, D.M., Serxner, L.E., Howk, D.J., Hudson, J. & Todaro, G.J. (1974) *Proc. Nat. Acad. Sci. U.S.A.*, **71**, 57 - 62.

Lowry, O.H., Rosebrough, N.J., Farr A.L. & Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.

Maia, J.C.C., Rougeon, F. & Chapeville, F. (1971) *FEBS Lett.*, **18**, 130 - 134.

Martin, R. & Ames, B. (1961) *J. Biol. Chem.*, **236**, 1372 - 1379.

Mondal, H., Galangher, R.E. & Gallo R.C. (1975) *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1194 - 1198.

Sedwick, W.D., Wang, T.S.F. & Korn, D. (1972) *J. Biol. Chem.*, **247**, 5026 - 5033.

Spadari, S. & Weissbach, A. (1974a) *J. Biol. Chem.*, **249**, 5809 - 5815.

Spadari, S. & Weissbach, A. (1974b) *J. Mol. Biol.*, **86**, 11 - 20.

Srivastava, B. J. S. (1974) *Concer Res.*, **34**, 1015 - 1026.

Szkudlarek, J., Radzikowski, Cz., Czarnomska, A., Bartoszewicz W. & Dux, K. (1976) *Int. J. Cancer* (in press).

Wang, T.S.F., Sedwick, W.D. & Korn, D. (1974) *J. Biol. Chem.*, **249**, 841 - 850.

Weissbach, A. (1975) *Cell*, **5**, 101 - 108.

Żmudzka, B., Janion, C. & Shugar, D. (1969) *Biochem. Biophys. Res. Commun.*, **37**, 895 - 902.

POLIMERAZY DNA KOMÓREK MYSIEJ BIAŁACZKI LBN/b

Streszczenie

W postmitochondrialnej frakcji komórek mysiej białaczki LBN/b stwierdzono obecność czterech frakcji o aktywności polimeraz DNA (I, II, III, IV). Frakcję I, na podstawie powinowactwa do jonowymieniaczy oraz zdolności do replikacji poli(A), poli(C) i poli(Cm) zakwalifikowano jako RNA-

zależną polimerazę DNA pochodzenia wirusowego. Frakcje II, III i IV, na podstawie powinowactwa do jonowymieniaczy, ciężaru cząsteczkowego, właściwości matrycowych, zależności aktywności od pH, stężenia NaCl i jonów dwuwartościowych oraz czułości na inhibicję *N*-etylomaleimidem, zakwalifikowano jako DNA-zależne polimerazy DNA β, α i γ.

Trzy frakcje, RNA-zależna polimeraza DNA, DNA-zależna polimeraza DNA β i γ włączały dTMP na matrycy poli(A) z primerem oligo(dT)$_{12-18}$. Mimo podobieństwa reakcji polimeraz β i γ z poli(A)·oligo(dT)$_{12-18}$ niektóre inne cechy tych enzymów sugerują, że reprezentują one dwa odrębne białka enzymatyczne.

Received 4 November, 1975.

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 1

RECENZJE KSIĄŻEK

COLLECTED TENTATIVE RULES AND RECOMMENDATIONS OF THE COMMISSION ON BIOCHEMICAL NOMENCLATURE, IUPAC - IUB. II wydanie, czerwiec 1975; The American Society of Biochemical Chemists, Inc., Bethesda; stron 149, cena $ 3.00.

Komisja Nomenklatury Biochemicznej Międzynarodowej Unii Chemii Czystej i Stosowanej (IUPAC) i Międzynarodowej Unii Biochemicznej (IUB) wydaje sukcesywnie tymczasowe wytyczne i zalecenia dotyczące nomenklatury poszczególnych działów biochemii.

Obecnie ukazało się w języku angielskim II wydanie zebranych tymczasowych wytycznych i zaleceń obejmujące materiały wydane do końca maja 1975 r.

Warto nadmienić, że *Polskie Słownictwo Biochemiczne* pod redakcją A. Morawieckiego (Monografie Biochemiczne Polskiego Towarzystwa Biochemicznego, nr 29; PWN, Warszawa 1974) uwzględnia zalecenia Komisji Nomenklatury Biochemicznej opublikowane do 1972 r.

Redakcja

W.B. QUAY. PINEAL CHEMISTRY IN CELLULAR AND PHYSIOLOGICAL MECHANISMS. Charles Thomas Publ., Springfield 1974; str. 430. cena $ 25.

Recenzowana książka należy do publikacji *Bannerstone Division of American Lectures in Living Chemistry*, serii redagowanej przez I. Newtona Kugelmassa, przeznaczonej dla osób pracujących w dziedzinie nauk biomedycznych.

Duże znaczenie omawianej książki polega w pierwszym rzędzie na tym, że jest to pierwsze monograficzne opracowanie fizjologii szyszynki, integrujące wyniki badań morfologicznych, biochemicznych i fizjologicznych w ścisłym znaczeniu tego słowa. Autor, profesor neuroendokrynologii Uniwersytetu Wisconsin, jest kompetentnym znawcą przedmiotu, czynnym badaczem biochemii szyszynki od lat około dwudziestu. Owocem jego badań jest ponad 60 publikacji z tej dziedziny wiedzy.

Książka, pomimo iż dotyczy szczegółowego zagadnienia, napisana jest w sposób mogący zainteresować nie tylko wąskich specjalistów, lecz również neurofizjologów, neurochemików i neuroanatomów. Autor wykazuje duże doświadczenie dydaktyczne; każdy rozdział kończy się podsumowującymi wnioskami. Ułatwia to znakomicie przyswojenie materiału, a nawet umożliwia zaznajomienie się z głównymi danymi bez szczegółowego czytania całego rozdziału, gdy jakiś temat jest dla czytelnika mniej ważny.

Ogromny materiał faktyczny dotyczy szyszynki głównie szczura, myszy i człowieka; opiera się on na ponad 1100 publikacjach i zestawiony jest w postaci dużej liczby (około 90) przejrzystych tablic i bardzo dobrych oryginalnych rysunków i schematów (około 70).

Książka podzielona jest na 14 rozdziałów. Dwa pierwsze — wprowadzające — poświęcone są omówieniu rozwoju embrionalnego szyszynki i jej morfologii. Ultrastrukturę pinealocytu, specyficznej komórki szyszynki, autor przedstawił na tle schematu podkreślającego podobieństwa i róż-

nice z neuronem. Pozostałe rozdziały obejmują zagadnienia metabolizmu różnych składników (np. lipidów, węglowodanów, aminokwasów, nukleotydów i kwasów nukleinowych, białek i peptydów, jak również składników nieorganicznych). Jedną z myśli przewodnich autora jest próba wychwycenia ewentualnie występujących cech charakterystycznych dla szyszynki, jak również porównywanie z metabolizmem innych tkanek, w pierwszym rzędzie mózgu.

Dotychczas nie udało się jeszcze uzyskać danych, które mogłyby wskazywać na cytochemiczną odrębność pinealocytów. Synteza amin biogennych przebiega bardzo podobnie w pinealocytach i w komórkach nerwowych. Wydaje się, że wysoka aktywność enzymu syntetyzującego melatoninę jest charakterystyczna dla szyszynki.

Szczególnie dużo uwagi autor poświęcił aminom indolowym w związku z osiągnięciami w tej dziedzinie w ostatnim dwudziestoleciu. Stwierdzono, że stężenie 5-hydroksytryptaminy w szyszynce jest o wiele większe niż w jakiejkolwiek innej tkance ssaków. Szereg badaczy przypisuje melatoninie i 5-metoksy-pochodnym rolę hormonów szyszynki, które działają na ośrodkowy układ nerwowy, przysadkę i na niektóre gruczoły wydzielania wewnętrznego i narządy rozrodcze. Większość badaczy, w tym również autor książki, wyrażają pogląd, że podwzgórze jest miejscem docelowym działania hormonu szyszynki, a wpływ na układ przysadka - narządy rozrodcze jest wtórny za pośrednictwem podwzgórza. Mniej natomiast badaczy sądzi (i te dane doświadczalne są mniej przekonywające), że występuje bezpośredni wpływ szyszynki na przysadkę i na obwodowe gruczoły wydzielania wewnętrznego. Można również spotkać się z poglądem, że metabolizm wątroby, a pośrednio i skład chemiczny krwi, podlega działaniu szyszynki.

W ostatnim rozdziale autor podsumował wyniki badań dokonanych różnymi technikami, starając się je zintegrować. Szczegółowo omówił także poglądy o roli szyszynki jako tzw. zegara wewnętrznego, regulującego cykl dobowy.

Ważnym elementem omawianej monografii jest wskazywanie przez autora braków w danych doświadczalnych, przedstawianie różnych poglądów poszczególnych autorów i wyraźne zaznaczanie, które poglądy są hipotetyczne. Dziwi natomiast bardzo skąpy rys historyczny.

Wartość książki podnosi jej bardzo ładna strona graficzna i przejrzyście zestawione piśmiennictwo.

Stella Niemierko

MTP International Review of Science. BIOCHEMISTRY OF NUCLEIC ACIDS. Biochemistry, Series One, vol. 6 (Consultant editors: H. L. Kornberg, D. C. Phillips; volume editor: K. Burton). Butterworths, London - University Park Press, Baltimore 1974; stron 364, cena £ 8.50.

Intensywny rozwój biologii molekularnej doprowadza do nagromadzenia ogromnego zasobu faktów i coraz większej specjalizacji w poszczególnych dziedzinach tej nauki. W skrajnych przypadkach zdarza się, że biochemicy uprawiający jedną z dziedzin biochemii nie rozumieją nawet terminologii używanej w innej dziedzinie tej samej dyscypliny.

Liczne artykuły przeglądowe, ukazujące się w prasie fachowej, nie są w stanie skutecznie przeciwdziałać tej sytuacji ani w przypadku specjalisty z jednej dziedziny, który chce poznać inną dziedzinę biologii molekularnej, ani tym bardziej w przypadku studentów, magistrantów czy doktorantów. Dlatego z uznaniem należy powitać takie wydawnictwa jak *MTP-Review of Science*, które podjęło próbę wydawania całych serii przeglądów różnych dziedzin nauki w formie tomów poświęconych określonemu zagadnieniu. Każdy tom składa się z szeregu artykułów, opracowanych w zasadzie przez różnych specjalistów. Ten chwyt wydawniczy, szeroko obecnie stosowany, przyśpiesza znacznie opracowanie tomu i zapewnia wysoki poziom fachowy artykułów.

W I Serii Biochemicznej (składającej się z 12 tomów) tom szósty dotyczy biochemii kwasów nukleinowych. Zawiera on 11 rozdziałów obejmujących najbardziej aktualne zagadnienia z zakresu biologii molekularnej DNA i RNA, od oznaczania sekwencji nukleotydów poprzez replikację i rekombinację aż do regulacji syntezy kwasów nukleinowych u *prokaryota* i *eukaryota*.

W pierwszym rozdziale K. Murray pisze o oznaczaniu sekwencji nukleotydów. Opisana jest ogólna metodyka postępowania i przykłady oznaczania sekwencji tRNA, RNA fagów oraz rybosomowego RNA. W części dotyczącej oznaczania sekwencji nukleotydów DNA wspomina się o możliwościach użycia oczyszczonych enzymów restrykcyjnych, które uzyskują coraz szersze zastosowanie.

Drugi rozdział dotyczy zależności właściwości DNA od określonej sekwencji nukleotydów. W pierwszej części tego rozdziału autorzy (R.D. Wells i R.M. Wartell) rozpatrują przykłady, dotyczące zarówno naturalnych jak i syntetycznych polinukleotydów, świadczące o tym, że konformacja oraz własności biologiczne DNA są determinowane przez sekwencje nukleotydów. Ten kierunek badań zmierza do rozstrzygnięcia pytania, czy regulacyjne *loci* DNA mają szczególną konformację, różniącą je od *loci* strukturalnych. Druga część rozdziału omawia problemy związane z przygotowywaniem syntetycznych polinukleotydów jako związków modelowych do badań strukturalnych.

Treść trzeciego rozdziału jest logiczną kontynuacją drugiego, chociaż tytuł "DNA chromosomów bakteryjnych i wirusowych" jest chyba zbyt ogólny. M.G. Smith pisze w nim o sposobach oznaczania rozkładu nukleotydów wzdłuż łańcucha, o oznaczaniu wielkości genomu i konfiguracji chromosomu bakteryjnego *in vivo*. Część rozdziału poświęcona operonom i transkryptonom jest potraktowana zbyt skrótowo i ogólnikowo i wskutek tego nie zawiera w zasadzie wartościowych informacji, ani dla specjalistów, ani dla studentów.

Czwarty rozdział jest poświęcony DNA *eukaryota* (E. Southern). Podkreśla on zasadnicze różnice istniejące pomiędzy genomem *prokaryota* i *eukaryota* i związane z tym odmienne metody używane w badaniach nad DNA organizmów wyższych. Autor poświęca sporo miejsca opisowi powtarzających się sekwencji, następnie omawia sekwencje kodujące i kończy opisem teorii budowy chromosomów organizmów wyższych.

W rozdziale piątym P.T. Emmerson pisze o replikacji DNA u *E. coli*. Problem jest przedstawiony w sposób zwięzły i bardzo jasny. Obejmuje zarówno opis polimeraz i innych białek biorących udział w elongacji łańcucha, jak i mechanizmy inicjacji i terminacji oraz wyniki genetycznych badań nad replikacją chromosomu bakterii.

Szósty rozdział (N. Symonds) dotyczy rekombinacji. Wespół z poprzednim rozdziałem stanowią one całość, obejmującą najważniejsze procesy molekularne zachodzące w DNA.

Kolejny, siódmy rozdział (A. Travers) poświęcony jest transkrypcji u bakterii. Obejmuje on opis struktury i funkcji polimerazy RNA, mechanizmu jej działania, jak też regulacji transkrypcji. Ostatnia część rozdziału zawiera wzmiankę o układach transkrypcji u organizmów wyższych. Tylko podstawowe wiadomości o działaniu polimerazy zostały przytoczone; pominięto szczegóły dotyczące terminacji i antyterminacji, zaś w opisie funkcji polimeraz w rozwoju fagów pominięto doskonałe modele opracowane na układach bakteriofaga lambda.

Uzupełnieniem, w pewnym stopniu, rozdziału 7 są rozdziały 9 i 10, w których odpowiednio N.C. Craig pisze o syntezie rybosomalnego RNA i S. Nishimura o budowie i syntezie tRNA.

Te trzy rozdziały obejmują najważniejsze problemy dotyczące mechanizmów syntezy i regulacji wytwarzania RNA w komórkach.

Ósmy rozdział dotyczy RNA-zależnej polimerazy DNA (P.S. Sarin i R.C. Gallo). Omówiono w nim właściwości odwrotnych transkryptaz różnych wirusów, mechanizmy reakcji katalizowanych przez transkryptazy oraz rolę tych enzymów w onkogenezie.

Ostatni — jedenasty — rozdział poświęcony jest kwasom nukleinowym chloroplastów. R.J. Ellis i M.R. Hartley omawiają w nim właściwości, funkcję i syntezę DNA oraz RNA chloroplastów.

Obecność tego rozdziału mimo woli podkreśla fakt braku artykułu dotyczącego kwasów nukleinowych mitochondriów. Problem ten nabiera ostatnio coraz większego znaczenia ze względu na możliwość połączenia badań biochemicznych i genetycznych nad mitochondriami u drożdży.

Zwraca także uwagę pominięcie problemu fizycznego mapowania DNA, które przyniosło tak istotne wyniki w ustaleniu map genetycznych plazmidów lub wirusów — takich jak adenowirus lub SV 40.

Niezależnie od tego szósty tom *MTP — International Review of Science* pt. *Biochemistry of Nucleic Acids* stanowi bardzo wartościową książkę, która zainteresuje zarówno specjalistów z dziedziny biologii molekularnej, jak i tych, którzy swą wiedzę w tej dziedzinie pragną rozszerzyć.

Michał Bagdasarian

MTP International Review of Science. SYNTHESIS OF AMINO ACIDS AND PROTEINS. Biochemistry, Series One, vol. 7 (Consultant editors: H.L. Kornberg, D.C. Phillips; volume editor: H.R.V. Arnstein). Butterworths, London - University Park Press, Baltimore 1975; stron 416, cena £ 8.50.

Omawiana książka, dotycząca syntezy aminokwasów i białek, składa się z 9 opracowań, z których każde stanowi oddzielny, bardzo starannie opracowany artykuł przeglądowy.

W pierwszym rozdziale H.E. Umbarger (*Purdue University, Indiana*) w sposób wyczerpujący przedstawia procesy biosyntezy aminokwasów w komórkach mikroorganizmów. Na uwagę zasługuje fakt, że autor nie ogranicza się jedynie do omówienia szlaków metabolicznych, ale wiele uwagi poświęca kontroli i regulacji tych procesów.

Wszystkie pozostałe rozdziały poświęcone są omówieniu różnych aspektów badań nad biosyntezą białka. Kolejno omówione są badania, które doprowadziły do zrozumienia molekularnych podstaw kodu genetycznego oraz mechanizmów jego odczytywania. I tak F. Kalousek i W. Konigsberg (*Yale University*) opracowali zagadnienia związane z aktywnością aminokwasów. Autorzy omawiają właściwości syntetaz aminoacylo-tRNA, metody izolowania i oczyszczania tych białek, ich rozmieszczenie w komórce oraz genetyczną regulację. Inicjacja biosyntezy białka omówiona została przez R.J. Jacksona (*University of Cambridge*). Przedstawione są eksperymentalne dowody na połączenie cyklu rybosomalnego z procesami inicjacji oraz udział formylometionylo-tRNA w tworzeniu kompleksu inicjującego. Na uwagę zasługuje fakt, że autor w szerokim zakresie omawia również mechanizm inicjacji oraz jego kontrolę w komórkach eukariotycznych.

J. Modolell i D. Vazquez (*Instituto de Biologia Celular, Madrid*) zajęli się molekularnymi podstawami reakcji wydłużania i terminacji łańcucha polipeptydowego. Kolejno omówione zostały procesy wiązania aminoacylo-tRNA, translokacji oraz mechanizm tworzenia wiązania peptydowego. Autorzy podkreślają również różnice występujące w omawianych procesach w komórkach bakteryjnych i eukariotycznych

W następnych rozdziałach omówione są kolejno: struktura i funkcja rybosomu (R.A. Cox i E. Godwin, *National Institute for Medical Research, London*), kod genetyczny (R.L. Watts i D.C. Watts, *Guy's Hospital Medical School, London*), odczytywanie mRNA w układach bezkomórkowych (J.B. Lingrel, *Medical Research Council, Cambridge*), hormonalna regulacja biosyntezy białka (K.L. Manchester, *University of the West Indies, Jamaica*) i wewnątrzkomórkowa organizacja procesów biosyntezy białka (H.N. Munro i P.M. Steinert, *Massachusetts Institute of Technology*).

Dwa ostatnie rozdziały zasługują na specjalną uwagę, gdyż stanowią jedną z pierwszych prób kompleksowego przedstawienia obecnej wiedzy o organizacji i mechanizmach kontrolujących procesy biosyntezy białka w wysoko zorganizowanych komórkach organizmów wielokomórkowych i wielotkankowych. Udział hormonów w regulacji szybkości syntezy białka, a także w pewnym ukierunkowaniu tych procesów, sugerowany był od dawna. Niemniej dopiero lata 60-te przyniosły doświadczalne dowody na wpływ hormonów na procesy transkrypcji i translacji. K.L. Manchester w sposób krytyczny omawia dotychczasowe badania, wskazując, że pewne nowe koncepcje będą konieczne dla zrozumienia tych bardzo złożonych procesów.

Munro i Steinert w artykule poświęconym procesom biosyntezy białka w komórkach zwierzęcych i roślinnych omawiają rolę rybosomów wolnych i związanych z endoplazmatycznym retikulum, oraz przedstawiają mechanizmy powstawania tych dwóch klas polirybosomów. Omawiane są w sposób dokładny i krytyczny hipotezy o różnej funkcji białek syntetyzowanych na wolnych i związanych polirybosomach. W rozdziale tym omówiono również pewną autonomię w zakresie biosyntezy białka takich organelli podkomórkowych jak mitochondria i chloroplasty.

Procesy biosyntezy białka są niezwykle skomplikowane. Wymagają one rybosomów, mRNA, wielu rodzajów tRNA i syntetaz aminoacylo-tRNA, czynników białkowych, ATP, GTP i całego szeregu związków niskocząsteczkowych, które tworzą odpowiednie środowisko. Około 150 makromolekuł musi reagować nawzajem ze sobą, aby mogła nastąpić synteza jednej cząsteczki białka lub nawet tylko jednego łańcucha polipeptydowego. Te niezwykle złożone procesy przedstawione są w recenzowanej książce w sposób bardzo dokładny. Poszczególne rozdziały zostały napisane przez naukowców, których nazwiska na stałe związane są z tą dziedziną biologii molekularnej. Niezwykła znajomość omawianych zagadnień pozwoliła poszczególnym autorom na wybranie z ogromnej ilości informacji jedynie rzeczy najbardziej istotnych i przedstawienie tych zagadnień w sposób przystępny, umożliwiając zrozumienie nowych poglądów przez szerokie rzesze biochemików. Na podkreślenie zasługuje również to, że każdy rozdział zaopatrzony jest w bogatą bibliografię.

Witold Sendecki

BIOCHEMISTRY OF HORMONES. H.V. Rickenberg, editor. MTP International Review of Science. Butterworths, London - University Park Press, Baltimore 1974; str. 342, cena £ 8.50.

W cennym wydawnictwie ciągłym „MTP - Międzynarodowy Przegląd Nauki", tom ósmy z cyklu „Biochemia - Seria 1" poświęcony został biochemii hormonów. Ogólnym zamierzeniem redaktorów-konsultantów (H.L. Kornberg i D.C. Phillips) jest przedstawienie czytelnikowi molekularnych aspektów wybranych kierunków współczesnej biochemii i biofizyki w ich powiązaniach międzydyscyplinarnych. Zadanie redaktora tomu „Biochemia hormonów" (H.V. Rickenberg) było trudniejsze niż redaktorów pozostałych tomów z tego cyklu. Biologia molekularna hormonów rozwija się od kilku lat tak dynamicznie, że dokonanie przeglądu całości tej dyscypliny w ramach jednego tomu jest chyba rzeczą niemożliwą, a opóźnienie w stosunku do aktualnego stanu wiedzy jest nieuniknione, zwłaszcza jeżeli zamierza się wydać, jak w przypadku recenzowanej książki, dzieło o wzorowej formie edytorskiej.

Trudności te dobrze ilustrują już pierwsze rozdziały książki, poczynając od artykułu o mechanizmie działania insuliny (J.N. Fain). Po latach wielu niepowodzeń, w których kolejno identyfikowano domniemany zasadniczy etap działania insuliny — transport glukozy, synteza RNA, synteza białka, synteza cAMP — badania skoncentrowały się na pierwszym topograficznie, a zapewne i czasowo, zjawisku — na wiązaniu insuliny przez swoiste receptory komórkowe. Wiązanie to, wraz z reakcjami bezpośrednio po nim następującymi, stanowi najbardziej interesującą — ale nie najobszerniejszą — część rozdziału o insulinie. W podobny sposób, aczkolwiek z szerszym uwzględnieniem udziału systemu cyklicznych nukleotydów w całokształcie reakcji wyzwalanej przez wiązany z powierzchnią komórki hormon, przedstawiono w drugim rozdziale mechanizm działania ACTH (G. Sayers, R.J. Beal i S. Seelig). Jednakże zarówno w rozdziale dotyczącym kalcytoniny i paratyreoidyny (J.F. Habener i H. D. Niall), jak i w rozdziale o hormonach części gruczołowej przysadki mózgowej (V. Schreiber) zagadnienie interakcji hormonu z jego receptorem i wczesne reakcje w domenie błony komórkowej traktowane są raczej fragmentarycznie. Niewątpliwie — wiedza nasza o nich w czasie pisania książki była znacznie mniej kompletna niż obecnie. Tym niemniej, zarysowujące się już wówczas ogólne prawidłowości w mechanizmie działania hormonów innych niż sterydowe można było lepiej uwypuklić. Jedną z dróg do tego celu byłoby uwzględnienie w treści książki badań nad mechanizmem działania glukagonu, modelu szczególnie dogodnego, prace nad którym były już w latach 1973/74 dobrze zaawansowane. Nie umniejsza to zresztą wartości rozdziału o hormonach przysadki, a zwłaszcza rozdziału o metabolizmie wapnia, mającego bardziej monograficzny charakter niż pozostałe.

Hormonom sterydowym poświęcono więcej niż połowę książki. O mechanizmie działania sterydów płciowych żeńskich piszą B.W. O'Malley i A.R. Means, a o mechanizmie receptorowym w działaniu androgenów — S. Liao, a więc autorzy, których prace w decydującym stopniu przyczyniły się do obecnego stanu wiedzy o hormonach sterydowych. Obydwa te rozdziały cechuje jasne i syntetyczne przedstawienie aktualnych (w r. 1972) wyników, jak również ich omówienie w szerszym kon-

tekście indukcji ekspresji genu przez hormony sterydowe. Wiele z poruszonych problemów stało się przedmiotem intensywnych eksperymentów w latach 1973/74, których jednak autorzy nie włączyli do swych tekstów. Kilka prac z r. 1973, dotyczących cytozolowego receptora sterydów (transformacja, oczyszczanie za pomocą chromatografii powinowactwa), znalazło się jako addendum do rozdziału o analogach hormonów sterydowych (H.B. Anstall), co bez wątpienia nie było najszczęśliwszym rozwiązaniem redaktorskim..

W odróżnieniu od tradycyjnych monografii itp. z dziedziny endokrynologii, recenzowana książka zawiera również rozdział o hormonach roślinnych (R. Cleland). Omówiono w nim szczegółowo auksyny, giberyliny i cytokininy, ale nie pominięto kwasu abscysowego, etylenu i hipotetycznego florigenu. Rozdział ten, bogaty w treść informacyjną, jest szczególnie interesujący dla tych, którzy zajmują się hormonami zwierzęcymi, gdyż pozwala na (spekulatywne) uogólnienia o ewolucji mechanizmów regulacyjnych w organizmach wielokomórkowych.

Książkę kończy bardzo zwięzły rozdział o zastosowaniu hodowli komórkowych i organotypowych do studiów nad hormonami (E.H. Macintyre). Nie został on pomyślany jako podręcznik laboratoryjny, a stanowi raczej syntetyczne zestawienie piśmiennictwa, które powinno okazać się użyteczne dla endokrynologa, zamierzającego te techniki eksperymentalne wykorzystać w swych badaniach. Szkoda, że nie zdecydowano się włączyć do treści książki podobnego rozdziału o radioimmunologicznych i receptorowych metodach oznaczania hormonów, stanowiących co najmniej tak samo ważne narzędzie w biologii molekularnej hormonów jak hodowle tkankowe.

Ogólna ocena wartości książki jest rzeczą trudną. Z jednej strony — wysoki poziom prezentacji wyników, krytyczna ale kreśląca perspektywy rozwoju ich interpretacja sprawiają, iż czyta się ją z przyjemnością i pożytkiem. Z drugiej zaś — opóźnienia w stosunku do aktualnego stanu wiedzy są czasem irytujące dla czytelnika śledzącego na bieżąco odnośne piśmiennictwo. Nie ulega jednak kwestii, że „Biochemia hormonów" jest wysoce użytecznym zbiorem informacji dla badacza, który zamierza podjąć prace nad mechanizmami molekularnymi działania hormonów.

Kazimierz Zakrzewski

MTP International Review of Science. BIOCHEMISTRY OF CELL DIFFERENTIATION. Biochemistry, Series One, vol. 9 (Consultant editors: H.L. Kornberg, D.C. Phillips; volume editor: J. Paul). Butterworths, London - University Park Press, Baltimore 1974; stron 380, cena £ 8.50.

Omawiana pozycja, dotycząca biochemii różnicowania komórek, zawiera 10 rozdziałów, z których każdy jest oddzielnym artykułem przeglądowym.

W pierwszym rozdziale J.W. Ashworth (*University of Essex*) przedstawia cykl życiowy, metabolizm i różnicowanie komórkowe prymitywnych organizmów eukariotycznych — Acrasiales. W drugim rozdziale E. Mohr (*University of Freiburg*) zajmuje się fotoregulacją poziomu enzymów roślinnych przez układ fitochromowy. Wykrycie indukcji i represji pewnych enzymów przez fitochrom umożliwia opis zjawiska fotomorfogenezy w kategoriach molekularnych. Prawdopodobnie większość enzymów podstawowego metabolizmu jest niewrażliwa na fitochrom — przykładem jest liaza izocytrynianowa, kluczowy enzym cyklu glioksalowego. Spośród indukowanych enzymów najlepiej poznano amoniako-liazę L-fenyloalaniny, kluczowy enzym tej drogi metabolizmu roślin, która prowadzi do syntezy flawonoidów.

W kolejnych dwóch rozdziałach przedstawiono pewne cechy wczesnego rozwoju zwierząt. J. Paul (*The Beatson Institute for Cancer Research, Glasgow*) omawia syntezę poszczególnych rodzajów RNA i białka w zapłodnionym jaju jeżowca. Brak syntezy białka w niezapłodnionym jaju tłumaczą dwie hipotezy: nieaktywnych polisomów i mRNA „zamaskowanego", t.j. połączonego z białkiem.

Artykuł H. Denisa (*Centre de Génétique Moléculaire, C.N.R.S.*) dotyczy syntezy kwasów nukleinowych podczas oogenezy i wczesnego rozwoju płazów. Szczegółowo została omówiona synteza chromosomalnego i pozachromosomalnego DNA oraz synteza RNA, głównie rRNA. Autor zwraca uwagę na amplifikację pozachromosomalnego DNA kodującego 18S i 28S rRNA. Celowość

biologiczna tej amplifikacji związana jest ze wzmożonym zapotrzebowaniem na rybosomy przy znacznie zwiększonej syntezie białka po zapłodnieniu.

P.A. Marks, R.A. Rifkind i A. Bank (*Columbia University, New York*) omawiają różnicowanie komórki erytroidalnej ssaków. Przedstawiono m.in. miejsca erytropoezy w rozwijającym się płodzie myszy, rodzaje i szybkość syntezy łańcuchów globiny oraz mRNA globiny. Wykazano, że wpływ erytropoetyny na wykształcenie się erytroblastu z jego prekursora jest związany z pojawianiem się aktywnego mRNA dla globiny.

A.R. Means i B.W.O'Malley (*Baylor College of Medicine, Houston*) zajmują się indukowanym przez estrogen różnicowaniem jajowodu niedojrzałej kury. Obserwowano zmiany histologiczne, wzmożoną proliferację, wzrost syntezy DNA, zmiany w chromatynie dotyczące białek niehistonowych, wzrost syntezy RNA, wzrost aktywności polimerazy I i II, syntezę białek specyficznych dla dojrzałej tkanki, jak owoalbumina, lizozym, dekarboksylaza ornityny. Przedstawiono hipotetyczny schemat kolejnych etapów działania hormonu na komórki tkanki docelowej.

Kolejny rozdział dotyczy syntezy i degradacji białka w tkankach zwierzęcych. R.T. Schimke (*Stanford University*) podaje przegląd stosowanych układów doświadczalnych i metod oraz model matematyczny zmian poziomu enzymu. Regulację aktywności omawia głównie na przykładzie oksygenazy tryptofanu i aminotransferazy tyrozyny. Autor podkreśla, że w badaniach molekularnych mechanizmów regulacji syntezy enzymu najbardziej miarodajna jest ocena ilościowa i wyizolowanie specyficznego mRNA oraz polisomów syntetyzujących specyficzne białko. Odrębnym zagadnieniem jest regulacja degradacji białka, szczególnie istotna u organizmów eukariotycznych.

F.C. Kafatos i R. Gelinas (*Harvard University*) przedstawiają zagadnienia stabilności mRNA i kontroli syntezy specyficznych białek w komórkach wysokozróżnicowanych. Omawiane są zależności między ilością specyficznego mRNA a syntezą białka, szybkość syntezy i degradacji mRNA. Wykazano dużą rozmaitość okresu półtrwania mRNA w komórkach eukariotycznych, przy czym mRNA dla białka charakterystycznego dla danej wysokozróżnicowanej komórki jest bardzo stabilny.

W.J. Rutter, M.I. Goldberg i J.C. Perriard (*University of California*) omawiają polimerazy RNA u organizmów prokariotycznych i eukariotycznych, ich własności, mechanizm działania i rolę w regulacji transkrypcji. Zwrócono uwagę na istotne zmiany w aktywności polimeraz, m.in. we wczesnym rozwoju embrionalnym i podczas indukcji hormonami sterydowymi. Przedstawiono hipotetyczny mechanizm specyficznej transkrypcji.

Ostatni rozdział poświęcony jest roli białek chromosomalnych w regulacji aktywności genów. A.J. Mac Gillivray i D. Rickwood (*The Beatson Institute for Cancer Research, Glasgow*) opisują szczegółowo izolowanie, budowę i aktywność metaboliczną histonów i białek niehistonowych w tkankach dojrzałych i rozwijających się. Przedstawiono liczne dane eksperymentalne wskazujące na rolę regulacyjną białek niehistonowych i rolę histonów w utrzymaniu struktury chromosomu.

Recenzowana książka stanowi próbę przedstawienia na poziomie molekularnym wielu zjawisk znanych w biologii rozwoju. Przybliżając czytelnikowi złożone procesy różnicowania jest niewątpliwie cenną i interesującą pozycją.

Ewa Dahlig

MODERN TRENDS IN HUMAN GENETICS - 2 (editor: A.E.H. Emery) Butterworths, London - Boston 1975; stron 496, cena £ 13.00.

W książce, która jest drugim tomem serii *The Modern Trends in Human Genetics*, przedstawiono obecny stan wiedzy na temat genetyki człowieka, ze szczególnym uwzględnieniem osiągnięć w badaniach klinicznych. Celem książki, jak pisze jej redaktor, prof. Alan E.H. Emery (*University of Edinburgh*), jest nie tylko podanie poznanych faktów, ale w równej mierze zwrócenie uwagi na właściwy kierunek dalszych badań i na te zagadnienia genetyki człowieka, które szczególnie wymagają szybkiego rozwiązania.

W omawianej książce przedstawiono techniki badania chromosomów, ich polimorfizmu oraz metody pozwalające na określenie rodzaju ich uszkodzenia. Zaprezentowano wstępną klasyfikację

błędów chromosomalnych, podejmując próbę przypisania poszczególnym błędom określonych zaburzeń rozwojowych. Zwrócono uwagę na możliwość szybkiego identyfikowania uszkodzeń struktury chromosomu poprzez badanie mejozy u osób normalnych i osób z uszkodzonymi chromosomami. Jeden z rozdziałów dotyczy uszkodzeń chromosomów indukowanych promieniowaniem, oraz wynikających stąd niebezpieczeństw.

Przedstawiono wyniki badań genetycznych i immunologicznych u chorych na leukemię, uwzględniając wpływ defektów genetycznych na funkcję leukocytów. Duży nacisk położono na badania dotyczące wpływu różnych czynników, m.in. alkoholizmu i podawania leków, na powstawanie wrodzonych wad metabolicznych.

Istotne znaczenie ma rozdział poświęcony genetycznie uwarunkowanym wadom wrodzonym i ich terapii. Poznanie przyczyn powstawania wad wrodzonych, znalezienie właściwych metod zapobiegania oraz ustalenie skutecznych metod leczenia — to najpilniejsze, zdaniem autorów, zadanie dla genetyków i lekarzy.

Szeroki zakres tematyki omawianej książki najlepiej obrazuje spis treści kolejnych rozdziałów:

Autosomal Abnormalities and the Banding Techniques - D.S. Borgaonkar (*John Hopkins University, Baltimore*)
Human Meiotic Studies - A.C. Chandley (*Western General Hospital, Edinburgh*)
Radiation-Induced Chromosome Damage and the Assessment of Genetic Risk - A.G. Searle (*Medical Research Council Radiobiology Unit, Harwell*)
Studies of Hepatic Phenotypes Expressed in Somatic Cell Hybrids: A Summary - G.J. Darlington (*Cornell Medical Centre, New York*) and F.H. Ruddle (*Yale University, New Haven*)
Genetics and Immunology in Human Leukaemia - R. Harris (*St. Mary's Hospital, Manchester*)
Genetic Defects of Leucocyte Function - J.A Raeburn (*Western General Hospital, Edinburgh*)
Mutation Rates in Man - P.M. Conneally (*Indiana University*)
Genetic Studies of Isolates - D.F. Roberts (*University of Newcastle upon Tyne*)
Genetic Disorders among the Jewish People - R.M. Goodman (*Tel-Aviv University*)
Environmental Factors in the Aetiology of Congenital Malformations in Man - J.R. Miller (*University of British Columbia, Vancouver*) and M. Yasuda (*Kyoto Prefectural School of Medicine*)
Disproportionate Dwarfism in the New-Born - P. Beighton (*Medical School, University of Cape Town*)
Analytical Aspects of Genetic Counselling - E.A. Murphy (*John Hopkins University, Baltimore*)
Artificial Insemination in the Human - D.W. Richardson (*Medical Research Council Unit of Reproduction Biology, Edinburgh*)
Treatment of Genetic Disease - H.L. Nadler and C.W. Booth (*North Western University, Chicago*).

Omawiana książka dostarcza wielu cennych wiadomości na temat badań genetycznych u ludzi oraz ich praktycznego wykorzystania w diagnostyce wielu schorzeń. Dostarcza bogatego materiału do refleksji i może być niewątpliwie źródłem inspiracji do podjęcia wielu nowych badań. Jest ona wartościową pozycją i na pewno będzie przyjęta z uznaniem tak przez genetyków i biochemików, jak i lekarzy.

Zofia Porembska

WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION. Twenty-sixth Report (Technical Report Series no. 565). WHO, Geneva 1975; stron 72, cena fr. szw. 7.-

Kolejne sprawozdanie Komitetu Ekspertów do spraw Standaryzacji w zakresie Biologii dotyczy standaryzacji preparatów hormonalnych do oznaczeń biologicznych i immunologicznych dla celów klinicznych. Obok opisu poszczególnych preparatów standardowych podano szczegółowo zasady przyjęte przy ustalaniu standardów.

Redakcja

COLLECTED
TENTATIVE RULES & RECOMMENDATIONS
of the
COMMISSION ON BIOCHEMICAL
NOMENCLATURE
IUPAC—IUB
SECOND EDITION - JUNE 1975,

vi + 149 pages — 8 1/2″ × 11″ paper bound

A compilation of all tentative rules and recommendations published through May 1975, edited only to eliminate superseded sections, and made available with the concurrence of the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and the Commission of Editors of Biochemical Journals of the International Union of Biochemistry.

$ 3.00 per copy; 20% discount on orders of ten or more copies to a single address—postpaid. Remittance in U.S. dollars must accompany order to—

American Society of Biological Chemists, Inc.
9650 Rockville Pike
Bethesda, Maryland 20014 USA

SUPPLEMENT 1 TO ENZYME NOMENCLATURE

Enzyme nomenclature: Recommendations (1972) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. Supplement 1: Corrections and additions (1975) will be published in Volume 429 of Biochimica et Biophysica Acta, pp. 1 - 45, appearing in March, 1976.

Reprints may be purchased from Elsevier Scientific Publishing Company, P.O. Box 1345, Amsterdam, The Netherlands, at the following rates (prices include postage)

1 or more copies:	Dfl. 15.00 each
10 or more copies:	Dfl. 14.00 each
50 or more copies:	Dfl. 12.00 each
100 or more copies:	Dfl. 11.00 each.

Payment must accompany orders.

Cena zł 25,—

ACTA BIOCHIMICA POLONICA

PRENUMERATA KRAJOWA

Cena prenumeraty krajowej

półrocznie	zł 50,—
rocznie	zł 100,—

Prenumeratę przyjmują Oddziały RSW „Prasa Książka-Ruch" oraz urzędy pocztowe i doręczyciele w terminach:

— do dnia 25 listopada na styczeń, I-szy kwartał, I-sze półrocze i cały rok następny,

— do dnia 10 każdego miesiąca (z wyjątkiem grudnia) poprzedzającego okres prenumeraty.

Jednostki gospodarki uspołecznionej, instytucje i organizacje społeczno-polityczne oraz wszelkiego rodzaju inne zakłady pracy, składają zamówienia w miejscowych Oddziałach RSW „Prasa-Książka-Ruch".

Zakłady pracy w miejscowościach, w których nie ma Oddziałów RSW oraz prenumeratorzy indywidualni, zamawiają prenumeratę w urzędach pocztowych lub u doręczycieli.

PRENUMERATA ZAGRANICZNA

Prenumeratę ze zleceniem wysyłki za granicę, która jest o 50% droższa od prenumeraty krajowej, przyjmuje Biuro Kolportażu Wydawnictw Zagranicznych RSW „Prasa-Książka-Ruch", ul. Wronia 23, 00-958 Warszawa.

Bieżące i archiwalne numery można nabyć lub zamówić we Wzorcowni Wydawnictw Naukowych PAN—Ossolineum—PWN, Pałac Kultury i Nauki (wysoki parter) 00-901 Warszawa oraz w księgarniach naukowych „Domu Książki".

A subscription order stating the period of time, along with the subscriber's name and address can be sent to your subscription agent or directly to Foreign Trade Enterprise Ars Polona-Ruch, 00-068 Warszawa, 7 Krakowskie Przedmieście, P.O. Box 1001, Poland. Please send payments to the account of Ars Polona-Ruch in Bank Handlowy S.A., 7 Traugutt Street, 00-067 Warszawa, Poland.

Acta bioch. pol. Vol. 23, No. 1, s. 1 - 84, Warszawa, styczeń - marzec 1976.

Indeks 35202